



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(54) Title:</b> METHODS FOR ENHANCEMENT OF PROTECTIVE IMMUNE RESPONSES EMPLOYING <i>LEISHMANIA</i> POLYPEPTIDES  <b>(57) Abstract</b>  Methods for eliciting or enhancing immune responses to antigens, including tumor antigens, and/or DNA vaccines are provided. The methods employ polypeptides or nucleic acid compositions that contain at least a biologically active portion of a <i>Leishmania braziliensis</i> or <i>Leishmania major</i> homologue of the eukaryotic initiation factor 4A, or a variant thereof. Such polypeptides and compositions are useful for enhancing or eliciting a patient's cellular and/or humoral immune response, for instance within methods for treating tumors.		

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## Description

### METHODS FOR ENHANCEMENT OF PROTECTIVE IMMUNE RESPONSES EMPLOYING *LEISHMANIA* POLYPEPTIDES

#### 5 TECHNICAL FIELD

The present invention relates generally to compounds and methods for enhancing immune responses in patients, as well as in isolated cells and cell cultures. The invention is more particularly related to compounds comprising all or a portion of a *Leishmania* antigen that is a homologue of the eukaryotic initiation factor 4A (eIF4A),  
10 and to the use of such compounds in vaccines for stimulating immune responses.

#### BACKGROUND OF THE INVENTION

Vaccines commonly induce immunity to an infection or a disease by generating an immune response in a patient to a specific antigen associated with the  
15 infection or disease. Modern techniques for the identification and use of appropriate antigens have the potential to lead to the testing and development of a large number of vaccines specific for common infections (including bacterial, viral and protozoan infections), as well as diseases such as cancer.

However, in many cases, purified antigens are weak immunogens,  
20 *i.e.*, the immune response generated by a specific antigen, while directed against the desired target, is not of a sufficient magnitude to confer immunity. In such cases, an immunomodulating agent, such as an adjuvant or immunostimulant, must be employed to enhance the immune response. Adjuvants are substances that enhance a specific immune response to an antigen when injected with the antigen or at the same site as the  
25 antigen. Such substances function by a variety of mechanisms, including (1) trapping the antigen, and releasing it slowly, (2) stimulating migration of cells to the injection site, (3) stimulating or trapping lymphocytes, or stimulating lymphocyte proliferation and (4) improving antigen dispersion within the patient's body. For example, oils, polymers, mineral salts and liposomes have been used as adjuvants in this regard. By  
30 comparison, immunostimulants are substances that induce a general, temporary increase in a patient's immune response, whether administered with the antigen or separately. Typical immunostimulants are bacterial, such as BCG (an attenuated strain of *Mycobacterium tuberculosis*) or a nonviable form of *Corynebacterium parvum*. By either mechanism, the adjuvant or immunostimulant serves to enhance the desired  
35 specific immune response by non-specific means.

A serious drawback of many of the adjuvants currently available is their toxicity. In general, the best adjuvants (*i.e.*, those that provide the greatest enhancement of the desired immune response) are also the most toxic. Thus, practitioners must continually balance the level of stimulation against the toxicity of the adjuvant.

5 Accordingly, there is a need in the art for the identification of compounds that provide a desired enhancement of specific immune responses, but with low levels of toxicity. The present invention fulfills these needs and further provides other related advantages.

## 10 SUMMARY OF THE INVENTION

Briefly stated, the present invention provides compounds and methods relating to the *Leishmania* antigen LbeIF4A or LmeIF4A, which is homologous to the eukaryotic ribosomal protein eIF4A. In one aspect of the invention, methods are provided for enhancing or eliciting an immune response to an antigen and/or an antigen  
15 encoded by a DNA vaccine in a patient, comprising administering to a patient an antigen and/or a DNA vaccine, and an LbeIF4A polypeptide comprising an amino acid sequence encoded by a DNA sequence selected from the group consisting of: (a) nucleotides 115-1323 of SEQ ID NO. 1; and (b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 115-1323 of SEQ ID NO. 1 under  
20 moderately stringent conditions, wherein the DNA sequence encodes a polypeptide that stimulates a Th1 immune response in a peripheral blood mononuclear cells obtained from a *Leishmania*-infected individual. In another aspect of the invention, methods are provided for enhancing or eliciting an immune response to an antigen and/or an antigen encoded by a DNA vaccine, in a patient, comprising administering to a patient an  
25 antigen and/or a DNA vaccine, and an LmeIF4A polypeptide comprising an amino acid sequence encoded by a DNA sequence selected from the group consisting of: (a) nucleotides 117 through 1325 of SEQ ID NO:3; and (b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 117 through 1325 of SEQ ID NO:3 under moderately stringent conditions, wherein the DNA sequence encodes a  
30 polypeptide that stimulates a Th1 immune response in peripheral blood mononuclear cells obtained from a *Leishmania*-infected individual.

In related aspects, the present invention provides methods for enhancing an immune response to an antigen and/or an antigen encoded by a DNA vaccine in a patient, comprising administering to a patient an antigen and/or a DNA vaccine, and an  
35 LbeIF4A polypeptide comprising amino acids 49-403 of SEQ ID NO: 2, or a variant thereof that differs only in conservative substitutions and/or modifications, or an antigen



and/or a DNA vaccine and an LmeIF4A polypeptide comprising amino acids 49-403 of SEQ ID NO: 4, or a variant thereof that differs only in conservative substitutions and/or modifications.

5 In another related aspect, methods are provided for enhancing an immune response in a biological sample, comprising contacting a biological sample with an antigen and/or an antigen encoded by a DNA vaccine, and an LbeIF4A polypeptide as described above, wherein the biological sample comprises cells selected from the group consisting of peripheral blood mononuclear cells, monocytes, B cells, dendritic cells, and combinations thereof.

10 In yet another related aspect, methods are provided for enhancing or eliciting an immune response in a biological sample, comprising contacting a biological sample with an LmeIF4A polypeptide as described above, wherein the biological sample comprises cells selected from the group consisting of peripheral blood mononuclear cells, monocytes, B cells, dendritic cells and combinations thereof.

15 In another aspect, methods are provided for enhancing an immune response to a tumor in a patient, comprising administering to a patient a tumor antigen or antigens and/or a DNA vaccine, and an LbeIF4A or an LmeIF4A polypeptide as described above.

20 Within further aspects, methods are provided for treating a tumor in a patient, comprising administering to a patient an LbeIF4A or LmeIF4A polypeptide, as described above.

25 Within each of the aspects noted above, as an alternative to utilizing an LbeIF4A or LmeIF4A polypeptide, one can utilize viral vectors or nucleic acid molecules (collectively, the "nucleic acid compositions") directing the expression of the polypeptide in patient cells infected or transfected with the nucleic acid compositions. The step of administering the nucleic acid composition may be performed *in vivo* or *ex vivo*, the latter including the subsequent administration of the infected/transfected cells. In addition, where an antigen or tumor antigen is administered, it will be evident that the nucleic acid composition may also be designed to direct the expression of such  
30 antigens (either on the same or different vectors or molecules).

35 Within further aspects, methods are provided for treating a Th2-mediated disease in a patient, comprising administering to a patient (a) an LeIF4A polypeptide; (b) a nucleic acid molecule directing the expression of an LeIF4A polypeptide in patient cells transfected with the nucleic acid molecule or (c) a viral vector directing the expression of an LeIF4A polypeptide in patient cells infected with the viral vector.

Th2-mediated diseases include asthma, allergy, Th2 mediated autoimmune disease and Helminth infection.

The present invention further provides methods for decreasing production of one or more Th2-associated cytokines in a patient, comprising  
5 administering to a patient (a) an LeIF4A polypeptide; (b) a nucleic acid molecule directing the expression of an LeIF4A polypeptide in patient cells transfected with the nucleic acid molecule or (c) a viral vector directing the expression of an LeIF4A polypeptide in patient cells infected with the viral vector. Within certain embodiments, the Th2-associated cytokine is IL-4 or IL-5.

10 Methods for stimulating or enhancing IL-18 production in a patient are also provided, comprising administering to a patient (a) an LeIF4A polypeptide; (b) a nucleic acid molecule directing the expression of an LeIF4A polypeptide in patient cells transfected with the nucleic acid molecule or (c) a viral vector directing the expression of an LeIF4A polypeptide in patient cells infected with the viral vector.

15 Within further aspects, methods are provided for enhancing or eliciting an immune response to an antigen in a biological sample, comprising contacting a biological sample with an LeIF4A polypeptide in combination with one or more Th1-associated cytokines.

The present invention also provides method for enhancing or eliciting an  
20 immune response to an antigen in a patient, comprising administering to a patient one or more Th1-associated cytokines in combination with (a) an LeIF4A polypeptide; (b) a nucleic acid molecule directing the expression of an LeIF4A polypeptide in patient cells transfected with the nucleic acid molecule or (c) a viral vector directing the expression of an LeIF4A polypeptide in patient cells infected with the viral vector. The Th1-  
25 associated cytokines may be IL-2, IL-12, IL-15 and/or IL-18.

These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

30

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 presents the results of Southern blot analysis of *Leishmania* spp. DNA, indicating that the *Leishmania* eIF4A homologue is conserved and that *L. braziliensis* genomic DNA contains at least two copies of LbeIF4A.

Figure 2 shows the results of an immunoblot analysis which demonstrates that LbeIF4A immune rabbit serum reacts with one dominant protein species of size ~45 kDa in different *Leishmania* species.

Figure 3 illustrates the ability of purified recombinant LbeIF4A to stimulate proliferation of PBMCs from *L. braziliensis*-infected individuals.

Figures 4A and 4B present the results obtained by analysis of cytokine mRNA expression patterns of PBMCs from patients with confirmed cases of *L. braziliensis* infection.

Figure 5 illustrates the supernatant levels of secreted IFN- $\gamma$  from PBMCs from *L. braziliensis*-infected individuals following stimulation with LbeIF4A or parasite lysate.

Figure 6 shows the levels of TNF- $\alpha$  detected in the supernatants of PBMCs from *L. braziliensis*-infected individuals following stimulation with LbeIF4A or parasite lysate.

Figure 7, Panels A-D, shows that LbeIF4A also stimulates patient PBMCs to secrete IL-12 in the cultured supernatant with a magnitude significantly higher than the IL-12 level stimulated by parasite lysate and that IL-10 inhibits this IL-12 production.

Figure 8, Panels A and B, demonstrates that in all patient PBMCs tested, IFN- $\gamma$  production was IL-12 dependent and inhibited by IL-10.

Figures 9A and 9B show that LbeIF4A stimulates IL-12 production in cultured human macrophages and adherent PBMCs.

Figure 10 indicates that LbeIF4A stimulates IL-12 p40 production in the human myeloid leukemia cell-line, THP-1, and synergizes with IFN- $\gamma$  to stimulate THP-1 cells to secrete IL-12.

Figure 11 presents results that indicate that lymph node cells of mice primed with LbeIF4A proliferate and secrete an almost exclusive Th1 cytokine profile.

Figure 12 demonstrates that LbeIF4A provides significant protection against *L. major* infection in an animal model recognized as having relevance to human disease.

Figure 13 illustrates the elicitation of anti-ovalbumin CTL using a representative LmeIF4A polypeptide.

Figure 14 illustrates the use of a representative LmeIF4A polypeptide as an adjuvant for the induction of antibodies specific for trinitrophenol.

Figure 15 shows the enhancement of anti-MUC-1 antibody production by a representative LmeIF4A polypeptide.

Figure 16 illustrates the enhancement of specific CTL activity by a representative LmeIF4A polypeptide in cultured cells.

Figure 17 shows the *in vitro* stimulation of CTL activity with IL-2, with and without an LmeIF4A polypeptide.

5           Figure 18 illustrates the induction of murine alloreactive CTL by an LmeIF4A polypeptide.

Figure 19 shows tumor regression following administration of tumor antigen and LmeIF4A polypeptide contained in microspheres.

10           Figure 20 shows tumor regression following administration of tumor antigen and LmeIF4A polypeptide contained in microspheres.

Figure 21 shows tumor regression following administration of soluble LmeIF4A polypeptide and tumor antigen contained in microspheres.

15           Figure 22 presents a comparison of the predicted amino acid sequences of *L. major* eIF4A (LmeIF), with the homologous proteins from *L. brazilienses* (LbeIF), mouse (MeIF) and human (HeIF). Positions of identical residues to LmeIF are shaded black. Boxed sequences represent identity between the mouse and human proteins that are distinct from the *Leishmania* homologue or conservative substitutions. Regions of similarity with conserved elements found in RNA helicases are indicated (I-VI). I and II (DEAD) represent specialized versions of the A and B motifs described in other ATP  
20           binding proteins. Cysteine residues are indicated by \* and potential N-linked glycosylation sites are underlined.

          Figures 23A-C illustrate the expression and purification of recombinant LmeIF. Figure 23A is a photograph of coomassie blue-stained 12% SDS-PAGE of *E. coli* lysates before (lane 1) and after (lane 2) induction with IPTG to express rLmeIF with  
25           6 amino-terminal histidine tag residues. rLmeIF following purification from the inclusion body by affinity chromatography on Ni-NTA column is shown in lane 3. Figure 23B is a photograph of coomassie blue-stained 12% SDS-PAGE of overlapping LeIF deletions. The recombinant clones were designed to encode the N-terminal half (26 kDa, residues 1-226, lane 1), the middle portion (16 kDa, residues 129-261, lane 2)  
30           and the C-terminal half (25 kDa, residues 196-403) of LeIF with six His-tag residues and the proteins purified over NiNTA resin. Protein molecular weight markers (lane M) are indicated to the left. Figure 23C is a schematic representation of the full length cDNA clone of *L. major* LeIF comprising of a 0.13 kb sequence of 5' untranslated (5'UTR) segment, an open reading frame of 1.209 kb coding for 403 amino acid long  
35           protein, and a 1.25 kb of 3' UTR terminating with a stretch of poly A tail. The arrows

below show the location and sizes of both the full-length and overlapping fragments of the LeIF constructs.

Figures 24A-C are graphs illustrating the analysis of the Th1/Th2 cytokine profile of draining lymph node cells from *L. major* infected BALB/c mice against rLeIF. Draining popliteal lymph node cells ( $2 \times 10^6$ /ml) isolated at (A) 10 and (B), 28 days of infection were stimulated *in vitro* with 10  $\mu$ g/ml each of rLeIF or SLA and the supernatants analyzed 72 hours later for the amount of IFN- $\gamma$  and IL-4. In Figure 24C, *L. major* infection sera from BALB/c mice (28 day post-infection) were analyzed and titrated for the presence of anti- rLeIF or rLmSTII specific antibody and compared with total promastigote lysate (SLA). Bound antibodies were detected with HRP conjugated goat anti-mouse IgG secondary antibody.

Figure 25 is a histogram illustrating the abrogation of the SLA-induced IL-4 secretion by LeIF. Lymph node cells were obtained from BALB/c mice infected with *L. major* (4 weeks post-infection) and were stimulated with SLA (10  $\mu$ g/ml) alone or in the presence of various concentration of LeIF. Cells were cultured for 3 days and supernatants were collected and assayed for the production of IL-4 and IFN- $\gamma$  by ELISA.

Figure 26 is a histogram illustrating the profile of T cell clones isolated from rLeIF or r8E-primed BALB/c mice. Mice were immunized subcutaneously with 70  $\mu$ g of the respective antigens without adjuvant. Ten days later, their lymph node cells were restimulated *in vitro* under limiting dilution with the same antigen, irradiated antigen presenting cells and IL-2. The resulting clones were re-stimulated with anti-CD3 mAb and supernatant cytokine patterns of the Th1- (IFN- $\gamma$ ), Th2- (IL-4) or Th0 (IFN- $\gamma$  and IL-4) were determined by ELISA. The result is presented as the percentage of clones expressing a Th1, Th2, or Th0 cytokine profile.

Figures 27A-C are histograms illustrating LeIF stimulation of the production of IFN- $\gamma$  by splenocytes from naive C3H- and Balb/c-SCID mice. In Figure 27A, splenocytes from SCID mice of both Balb/c and C3H background were cultured at  $2 \times 10^6$  per well and stimulated with 10  $\mu$ g/ml of the indicated antigen. Supernatants were harvested at 12, 24, and 72 hours and assayed for the production of IFN- $\gamma$ . In Figure 27B assays were performed as above using C3H SCID splenocytes in the presence or absence of anti-IL-12 antibody. Supernatants were harvested at 72 hours. In Figure 27C, stimulation was performed using three overlapping LeIF recombinants comprising amino acid residues 1-226, 129-261 and 196-403 at 2.5, 5.0, and 10  $\mu$ g/ml in splenocyte cultures from C3H SCID mice. As control, LPS was used at two concentrations, 100 ng and 1  $\mu$ g/ml.

Figure 28 is a photograph depicting the electrophoresis of RT-PCR experiments to determine the presence of various cytokines in SCID mouse splenocytes cultured for 24 hours in the absence (-) or presence (+) of LeIF(10 µg/ml). Primers were specific for β-actin as a control, or for IFN-γ, IL-18 or IL-10, as indicated.

5           Figure 29 is a graph showing the level of IFN-γ in SCID mouse splenocytes stimulated for 72 hours with varying amounts of IL-18 as indicated, in the presence or absence of LeIF (10 µg/ml).

          Figure 30 is a graph showing the level of IFN-γ in SCID mouse splenocytes stimulated for 72 hours with varying amounts of IL-18 as indicated, in the  
10   presence or absence of LeIF (10 µg/ml), IL-15 (ng/ml) or both.

          Figure 31 is a graph showing the level of IFN-γ in SCID mouse splenocytes stimulated for 72 hours with varying amounts of IL-18 as indicated, in the presence or absence of LeIF (10 µg/ml), IL-15 (100 ng/ml) or both.

          Figure 32 is a graph illustrating the cytotoxic activity (expressed as %  
15   Specific Lysis) of SCID mouse splenocytes stimulated with IL-15 (100 ng/ml), IL-12 (10 U/ml) or both in the presence or absence of LeIF (10 µg/ml) at varying effector:target ratios, as indicated. The target cells were YAC-1 cells.

          Figure 33 is a graph illustrating the cytotoxic activity (expressed as %  
20   Specific Lysis) of SCID mouse splenocytes stimulated with IL-18 (100 ng/ml), IL-12 (10 U/ml) or both in the presence or absence of LeIF (10 µg/ml) at varying effector:target ratios, as indicated. The target cells were YAC-1 cells.

#### DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention is generally directed to the  
25   enhancement of immune responses, which may be humoral and/or cell-mediated, in a patient or cell culture. Within the context of this invention, an immune response to an antigen, including an immunostimulating antigen (*i.e.*, an antigen against which a patient raises an immune response), may be initiated or enhanced by administering to the patient the antigen and one or more LbeIF4A-derived or LmeIF4A-derived  
30   polypeptides as described herein. Antigens and immunostimulating antigens are in general protein molecules and include molecules derived from viruses, such as HIV, HBV, influenza virus, respiratory syncytial virus, bacteria, such as *Hemophilus influenza*, *Pneumococcus pneumoniae*, and parasites, such as *Leishmania*, and *Trypanosoma*. In addition, an immune response to a tumor may be enhanced or elicited  
35   by administering to the patient a tumor antigen (*i.e.*, an antigen that stimulates an immune response (*e.g.*, CTL) to a tumor). Within the context of this invention, tumor

antigens include virally encoded molecules, MAGE-1, Her-2, PSA, and other molecules. Accordingly, the methods of this invention involve the co-administration of a specific antigen or immunostimulating antigen and an LeIF4A-derived polypeptide as disclosed herein. A tumor may also be treated by administering to the patient an  
5 LbeIF4A or LmeIF4A polypeptide in the absence of such an exogenously administered tumor antigen.

The LbeIF4A and LmeIF4A polypeptides of the present invention may also be used to elicit or enhance an immune response to an antigen encoded by a DNA vaccine. DNA vaccines encode one or more immunostimulating antigens, such that the  
10 antigen is generated *in situ*. For instance, the DNA vaccine may encode a tumor antigen and, optionally, an LeIF4A-derived polypeptide as described herein. In such vaccines, the DNA may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems, bacteria and viral expression systems. Appropriate nucleic acid expression systems contain the necessary  
15 DNA sequences for expression in the patient (such as a suitable promoter). Bacterial delivery systems involve the administration of a bacterium (such as *Bacillus-Calmette-Guerrin*) that expresses an epitope of a prostate cell antigen on its cell surface. The DNA may be introduced using a viral expression system (*e.g.*, vaccinia or other pox virus, retrovirus, or adenovirus), which may involve the use of a non-pathogenic  
20 (defective), replication competent virus. Suitable systems are disclosed, for example, in Fisher-Hoch et al., *PNAS* 86:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci.* 569:86-103, 1989; Flexner et al., *Vaccine* 8:17-21, 1990; U.S. Patent Nos. 4,603,112, 4,769,330, and 5,017,487; WO 89/01973; U.S. Patent No. 4,777,127; GB 2,200,651; EP 0,345,242; WO 91/02805; Berkner, *Biotechniques* 6:616-627, 1988; Rosenfeld et al., *Science* 252:431-434, 1991; Kolls et al., *PNAS* 91:215-219, 1994; Kass-Eisler et al., *PNAS* 90:11498-11502, 1993; Guzman et al., *Circulation* 88:2838-2848, 1993; and Guzman et al., *Cir. Res.* 73:1202-1207, 1993. Techniques for incorporating DNA into such expression systems are well known to those of ordinary skill in the art. The DNA may also be "naked," as described, for example, in published PCT application  
30 WO 90/11092, and Ulmer et al., *Science* 259:1745-1749, 1993, reviewed by Cohen, *Science* 259:1691-1692, 1993. The uptake of naked DNA may be increased by coating the DNA onto biodegradable beads, which are efficiently transported into the cells.

The compounds of this invention generally comprise a polypeptide that stimulates a Th1 or CTL (cytotoxic T lymphocyte) immune response in peripheral  
35 blood mononuclear cells (PBMCs). In particular, polypeptides comprising all or a stimulatory portion of a *Leishmania braziliensis* or *Leishmania major* homologue of the

eukaryotic ribosomal protein eIF4A are disclosed. Such proteins may be referred to herein as LbeIF4A and LmeIF4A, or as LbeIF and LmeIF, respectively. As used herein, the term "PBMCs" refers to preparations of nuclear cells that are present in peripheral blood. The term "polypeptide," in the context of this invention, encompasses amino acid chains of any length, including full length proteins and portions thereof, wherein amino acid residues are linked by covalent peptide bonds. Therefore, an "LbeIF4A polypeptide" comprises LbeIF4A, or a portion or other variant thereof that retains stimulatory activity. Similarly, an "LmeIF4A polypeptide" comprises LmeIF4A, or a portion or other variant thereof that retains stimulatory activity. As used herein, "LeIF4A" or "LeIF" refers to either LbeIF4A or LmeIF4A. Although LbeIF4A is described herein for exemplary purposes, within the context of this invention, LmeIF4A, portions thereof, and variants of the polypeptide (or portions thereof) may also be used. An LeIF4A polypeptide may consist entirely of one or more stimulatory portions of LeIF4A, or the stimulatory portion(s) may be supplied in the context of a larger protein that contains additional LeIF4A sequences and/or amino acid sequences heterologous to LeIF4A. Preferably, the polypeptides are substantially free of contaminating endogenous materials.

The polypeptides of the present invention include variants of LbeIF4A or LmeIF4A that retain the ability to stimulate a Th1 or CTL immune response in PBMCs. Such variants include various structural forms of the primary protein. Due to the presence of ionizable amino and carboxyl groups, for example, a LbeIF4A polypeptide may be in the form of an acidic or basic salt, or may be in neutral form. Individual amino acid residues may also be modified by oxidation or reduction.

Variants within the scope of this invention also include polypeptides in which the primary amino acid structure of LeIF4A or a fragment thereof is modified by forming covalent or aggregative conjugates with other polypeptides or chemical moieties such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives may be prepared, for example, by linking particular functional groups to amino acid side chains or at the N- or C-termini. Alternatively, for derivatives in which a polypeptide is joined to a LeIF4A polypeptide, a fusion protein may be prepared using recombinant DNA techniques, as described below. In one such embodiment, the LeIF4A polypeptide may be conjugated to a signal (or leader) polypeptide sequence at the N-terminal region of the protein which co-translationally or post-translationally directs transfer of the protein from its site of synthesis to its site of function inside or outside of the cell membrane or wall (e.g., the yeast  $\alpha$ -factor leader).



Protein fusions within the present invention may also comprise peptides added to facilitate purification or identification of LeIF4A polypeptides (*e.g.*, poly-His). For example, the peptide described by Hopp et al., *Bio/Technology* 6:1204 (1988) is a highly antigenic peptide that can be used to facilitate identification. Such a peptide provides an epitope reversibly bound by a specific monoclonal antibody, enabling rapid assay and facile purification of expressed recombinant protein. The sequence of Hopp et al. is also specifically cleaved by bovine mucosal enterokinase, allowing removal of the peptide from the purified protein. Fusion proteins capped with such peptides may also be resistant to intracellular degradation in *E. coli*.

Protein fusions encompassed by this invention further include, for example, LeIF4A polypeptides linked to an immunoglobulin Fc region. If LbeIF4A fusion proteins are made with both heavy and light chains of an antibody, it is possible to form a protein oligomer with as many as four LbeIF4A protein regions. Also within the scope of the present invention are LbeIF4A polypeptides linked to a leucine zipper domain. Leucine zipper domains are described, for example, in published PCT Application WO 94/10308. LbeIF4A polypeptides comprising leucine zippers may, for example, be oligomeric, dimeric or trimeric. All of the above protein fusions may be prepared by chemical linkage or as fusion proteins, as described below.

Preferred protein fusions include polypeptides that comprise sequences useful for stimulating immunity to infectious pathogens (*e.g.*, antigens). Such sequences may be derived, for example, from viruses, tumor cells, parasites or bacteria.

The present invention also includes LeIF4A polypeptides with or without associated native-pattern glycosylation. Polypeptides expressed in yeast or mammalian expression systems may be similar to or slightly different in molecular weight and glycosylation pattern than the native molecules, depending upon the expression system. For instance, expression of DNA encoding LbeIF4A polypeptides in bacteria such as *E. coli* provides non-glycosylated molecules. N-glycosylation sites of eukaryotic proteins are characterized by the amino acid triplet Asn-A<sub>1</sub>-Z, where A<sub>1</sub> is any amino acid except Pro, and Z is Ser or Thr. Variants of LbeIF4A polypeptides having inactivated N-glycosylation sites can be produced by techniques known to those of ordinary skill in the art, such as oligonucleotide synthesis and ligation or site-specific mutagenesis techniques, and are within the scope of this invention. Alternatively, N-linked glycosylation sites can be added to a LbeIF4A polypeptide.

The polypeptides of this invention also include variants of LeIF4A polypeptides that have an amino acid sequence different from the native LeIF4A protein because of one or more deletions, insertions, substitutions or other modifications. Such

variants should be substantially homologous to the native LeIF4A and should retain the ability to stimulate a Th1 or CTL immune response in PBMCs. "Substantial homology," as used herein, refers to amino acid sequences that may be encoded by DNA sequences that are capable of hybridizing under moderately stringent conditions to a naturally occurring DNA sequence encoding, for instance, LbeIF4A. Suitable moderately stringent conditions include prewashing in a solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing at 50°C-65°C, 5 X SSC, overnight; followed by washing twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1% SDS). Such hybridizing DNA sequences are also within the scope of this invention. The effect of any such modifications on the activity of a LbeIF4A polypeptide may be readily determined by analyzing the ability of the mutated LbeIF4A peptide to induce a Th1 or CTL response using, for example, any of the methods described herein. A preferred variant of LbeIF4A is the *Leishmania major* homologue of LbeIF4A (LmeIF4A).

Generally, amino acid substitutions should be made conservatively; *i.e.*, a substitute amino acid should replace an amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydrophobic nature of the polypeptide to be substantially unchanged. In general, the following groups of amino acids represent conservative changes: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. Variants within the scope of this invention may also, or alternatively, contain other modifications, including the deletion or addition of amino acids, that have minimal influence on the stimulatory properties, secondary structure and hydrophobic nature of the polypeptide. In general, fragments of LeIF4A may be constructed by deleting terminal or internal residues or sequences. Additional guidance as to suitable modifications may be obtained by a comparison of the sequence of LeIF4A to the sequences and structures of other eIF4A family members. For example, terminal or internal residues or sequences of LeIF4A not needed for biological activity may be deleted. Cysteine residues may be deleted or replaced with other amino acids to prevent formation of incorrect intramolecular disulfide bridges upon renaturation. Other approaches to mutagenesis involve modification of adjacent dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease activity is present.

An LeIF4A full length protein may generally be obtained using a genomic or cDNA clone encoding the protein. A genomic sequence that encodes full length LbeIF4A is shown in SEQ ID NO:1, and the deduced amino acid sequence is

presented in SEQ ID NO:2. A genomic sequence that encodes full length LmIF4A is shown in SEQ ID NO:3 and the deduced amino acid sequence in SEQ ID NO:4. Such clones may be isolated by screening an appropriate *Leishmania braziliensis* or *Leishmania major* expression library for clones that express antigens that react with sera

5 from a patient afflicted with mucosal leishmaniasis, and then analyzing the reactive antigens for the ability to stimulate proliferative responses and preferential Th1 cytokine production in patient T cell assays or for the ability to stimulate a CTL response in patient T cells. The library preparation and screen may generally be performed using methods known to those of ordinary skill in the art, such as methods described in

10 Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., 1989, which is incorporated herein by reference. Briefly, a bacteriophage expression library may be plated and transferred to filters. The filters may then be incubated with serum and a detection reagent. In the context of this invention, a "detection reagent" is any compound capable of binding to

15 the antibody-antigen complex, which may then be detected by any of a variety of means known to those of ordinary skill in the art. Typical detection reagents contain a "binding agent," such as Protein A, Protein G, IgG or a lectin, coupled to a reporter group. Preferred reporter groups include enzymes, substrates, cofactors, inhibitors, dyes, radionuclides, luminescent groups, fluorescent groups and biotin. More

20 preferably, the reporter group is horseradish peroxidase, which may be detected by incubation with a substrate such as tetramethylbenzidine or 2,2'-azino-di-3-ethylbenzthiazoline sulfonic acid. Plaques containing genomic or cDNA sequences that express a protein which binds to an antibody in the serum are isolated and purified by techniques known to those of ordinary skill in the art. Appropriate methods may be

25 found, for example, in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., 1989.

Patient T cell assays may generally be performed by treating patient PBMCs with the reactive antigens and analyzing the cells for a suitable response. For example, the PBMC supernatant may be assayed for the level of secreted cytokines.

30 Preferably, the cytokine assayed is interferon- $\gamma$ , interleukin-2, interleukin-12 (either the p40 subunit or biologically active p70), interleukin-1 or tumor necrosis factor- $\alpha$ . The cytokines interleukin-4 and interleukin-10 may also be assayed, since the levels of these representative Th2-type cytokines generally decrease in response to treatment with a polypeptide as described herein. Cytokines may be assayed, for example, using

35 commercially available antibodies specific for the cytokine of interest in an ELISA format, with positive results determined according to the manufacturer's instructions.

Suitable antibodies may be obtained, for example, from Chemicon, Temucula, CA and PharMingen, San Diego, CA. Alternatively, the treated PBMCs may be assayed for mRNA encoding one or more of the cytokines interferon- $\gamma$ , interleukin-2, interleukin-12 p40 subunit, interleukin-1 or tumor necrosis factor- $\alpha$ , or the PBMCs may be assayed for a proliferative response as described herein. Alternatively, cytokines may be measured by testing PBMC supernatants for cytokine-specific biological activities.

Variants of LeIF4A that retain the ability to stimulate a Th1 immune response in PBMCs may generally be identified by modifying the sequence in one or more of the aspects described above and assaying the resulting polypeptide for the ability to stimulate a Th1 response. Such assays may generally be performed by treating patient PBMCs with the modified polypeptide and assaying the response, as described above. Naturally occurring variants of LeIF4A may also be isolated from other *Leishmania* species by, for example, screening an appropriate cDNA or genomic library with a DNA sequence encoding LeIF4A or a variant thereof.

The above-described sequence modifications may be introduced using standard recombinant techniques or by automated synthesis of the modified polypeptide. For example, mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analogue having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide a gene in which particular codons are altered according to the substitution, deletion, or insertion required. Exemplary methods of making the alterations set forth above are disclosed by Walder et al., *Gene* 42:133, 1986; Bauer et al., *Gene* 37:73, 1985; Craik, *BioTechniques*, January 1985, 12-19; Smith et al., *Genetic Engineering: Principles and Methods*, Plenum Press, 1981; and U.S. Patent Nos. 4,518,584 and 4,737,462.

Mutations in nucleotide sequences constructed for expression of such LeIF4A polypeptides must, of course, preserve the reading frame of the coding sequences and preferably will not create complementary regions that could hybridize to produce secondary mRNA structures, such as loops or hairpins, which would adversely affect translation of the receptor mRNA. Although a mutation site may be predetermined, it is not necessary that the nature of the mutation *per se* be predetermined. For example, in order to select for optimum characteristics of mutants

at a given site, random mutagenesis may be conducted at the target codon and the expressed LeIF4A protein mutants screened for the desired activity.

Not all mutations in a nucleotide sequence which encodes a LeIF4A protein will be expressed in the final product. For example, nucleotide substitutions  
5 may be made to enhance expression, primarily to avoid secondary structure loops in the transcribed mRNA (see, e.g., European Patent Application 75,444A), or to provide codons that are more readily translated by the selected host, such as the well-known *E. coli* preference codons for *E. coli* expression.

The polypeptides of the present invention, both naturally occurring and  
10 modified, are preferably produced by recombinant DNA methods. Such methods include inserting a DNA sequence encoding a LeIF4A polypeptide into a recombinant expression vector and expressing the DNA sequence in a recombinant microbial, mammalian or insect cell expression system under conditions promoting expression. DNA sequences encoding the polypeptides provided by this invention can be assembled  
15 from cDNA fragments and short oligonucleotide linkers, or from a series of oligonucleotides, to provide a synthetic gene which is capable of being inserted in a recombinant expression vector and expressed in a recombinant transcriptional unit.

Recombinant expression vectors contain a DNA sequence encoding a LeIF4A polypeptide operably linked to suitable transcriptional or translational  
20 regulatory elements derived from mammalian, microbial, viral or insect genes. Such regulatory elements include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation, as described in detail below. An origin of replication and a selectable marker to facilitate recognition  
25 of transformants may additionally be incorporated.

DNA regions are operably linked when they are functionally related to each other. For example, DNA for a signal peptide (secretory leader) is operably linked to DNA for a polypeptide if it is expressed as a precursor which participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it  
30 controls the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous and, in the case of secretory leaders, in reading frame. DNA sequences encoding LeIF4A polypeptides which are to be expressed in a microorganism will preferably contain no introns that could prematurely terminate transcription of  
35 DNA into mRNA.

Expression vectors for bacterial use may comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed. *E. coli* is typically transformed using derivatives of pBR322, a plasmid derived from an *E. coli* species (Bolivar et al., *Gene* 2:95, 1977). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells.

Promoters commonly used in recombinant microbial expression vectors include the  $\beta$ -lactamase (penicillinase) and lactose promoter system (Chang et al., *Nature* 275:615, 1978; and Goeddel et al., *Nature* 281:544, 1979), the tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res.* 8:4057, 1980; and European Patent Application 36,776) and the tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, p.412, 1982). A particularly useful bacterial expression system employs the phage  $\lambda$   $P_L$  promoter and cI857ts thermolabile repressor. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the  $\lambda$   $P_L$  promoter include plasmid pHUB2, resident in *E. coli* strain JMB9 (ATCC 37092) and pPLc28, resident in *E. coli* RR1 (ATCC 53082).

Suitable promoter sequences in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.* 255:2073, 1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.* 7:149, 1968; and Holland et al., *Biochem.* 17:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., European Patent Application 73,657.

Preferred yeast vectors can be assembled using DNA sequences from pBR322 for selection and replication in *E. coli* (Amp<sup>r</sup> gene and origin of replication) and yeast DNA sequences including a glucose-repressible ADH2 promoter and  $\alpha$ -factor secretion leader. The ADH2 promoter has been described by Russell et al. (*J. Biol. Chem.* 258:2674, 1982) and Beier et al. (*Nature* 300:724, 1982). The yeast  $\alpha$ -factor leader, which directs secretion of heterologous proteins, can be inserted between the promoter and the structural gene to be expressed (see, e.g., Kurjan et al., *Cell* 30:933,

1982; and Bitter et al., *Proc. Natl. Acad. Sci. USA* 81:5330, 1984). The leader sequence may be modified to contain, near its 3' end, one or more useful restriction sites to facilitate fusion of the leader sequence to foreign genes. The transcriptional and translational control sequences in expression vectors to be used in transforming vertebrate cells may be provided by viral sources. For example, commonly used promoters and enhancers are derived from polyoma, adenovirus 2, simian virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide the other genetic elements required for expression of a heterologous DNA sequence. The early and late promoters are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers et al., *Nature* 273:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the *Hind* III site toward the *Bgl* II site located in the viral origin of replication is included. Further, viral genomic promoter, control and/or signal sequences may be utilized, provided such control sequences are compatible with the host cell chosen. Exemplary vectors can be constructed as disclosed by Okayama and Berg, *Mol. Cell. Biol.* 3:280, 1983.

A useful system for stable high level expression of mammalian receptor cDNAs in C127 murine mammary epithelial cells can be constructed substantially as described by Cosman et al. (*Mol. Immunol.* 23:935, 1986). A preferred eukaryotic vector for expression of LbIF4A protein DNA is pDC406 (McMahan et al., *EMBO J.* 10:2821, 1991), and includes regulatory sequences derived from SV40, human immunodeficiency virus (HIV), and Epstein-Barr virus (EBV). Other preferred vectors include pDC409 and pDC410, which are derived from pDC406. pDC410 was derived from pDC406 by substituting the EBV origin of replication with sequences encoding the SV40 large T antigen. pDC409 differs from pDC406 in that a *Bgl* II restriction site outside of the multiple cloning site has been deleted, making the *Bgl* II site within the multiple cloning site unique.

A useful cell line that allows for episomal replication of expression vectors, such as pDC406 and pDC409, which contain the EBV origin of replication, is CV-1/EBNA (ATCC CRL 10478). The CV-L/EBNA cell line was derived by transfection of the CV-1 cell line with a gene encoding Epstein-Barr virus nuclear antigen-I (EBNA-1) and constitutively express EBNA-1 driven from human CMV immediate-early enhancer/promoter.



Transformed host cells are cells which have been transformed or transfected with expression vectors constructed using recombinant DNA techniques and which contain sequences encoding a LeIF4A polypeptide of the present invention. Transformed host cells may express the desired LeIF4A polypeptide, but host cells  
5 transformed for purposes of cloning or amplifying LeIF4A DNA do not need to express the LeIF4A protein. Expressed LeIF4A proteins will preferably be secreted into the culture supernatant, depending on the DNA selected, but may also be deposited in the cell membrane.

Suitable host cells for expression of recombinant proteins include  
10 prokaryotes, yeast or higher eukaryotic cells under the control of appropriate promoters. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or *Bacilli*. Higher eukaryotic cells include established cell lines of insect or mammalian origin as described below. Cell-free translation systems could also be employed to produce LbeIF4A proteins using RNAs derived from the DNA constructs disclosed  
15 herein. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described, for example, by Pouwels et al., *Cloning Vectors: A Laboratory Manual*, Elsevier, New York, 1985.

Prokaryotic expression hosts may be used for expression of LeIF4A polypeptides that do not require extensive proteolytic and disulfide processing.  
20 Prokaryotic expression vectors generally comprise one or more phenotypic selectable markers, for example a gene encoding proteins conferring antibiotic resistance or supplying an autotrophic requirement, and an origin of replication recognized by the host to ensure amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and various  
25 species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although other hosts may also be employed.

Recombinant LeIF4A polypeptides may also be expressed in yeast hosts, preferably from the *Saccharomyces* species, such as *S. cerevisiae*. Yeast of other genera, such as *Pichia* or *Kluyveromyces* may also be employed. Yeast vectors will  
30 generally contain an origin of replication from the 2 $\mu$  yeast plasmid or an autonomously replicating sequence (ARS), a promoter, DNA encoding the LeIF4A polypeptide, sequences for polyadenylation and transcription termination and a selection gene. Preferably, yeast vectors will include an origin of replication and selectable marker permitting transformation of both yeast and *E. coli*, e.g., the ampicillin resistance gene  
35 of *E. coli* and the *S. cerevisiae* *trp1* gene, which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, and a promoter derived



from a highly expressed yeast gene to induce transcription of a structural sequence downstream. The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

5                   Suitable yeast transformation protocols are known to those of skill in the art. An exemplary technique described by Hind et al. (*Proc. Natl. Acad. Sci. USA* 75:1929, 1978), involves selecting for *Trp*<sup>+</sup> transformants in a selective medium consisting of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 mg/ml adenine and 20 mg/ml uracil. Host strains transformed by vectors comprising the  
10 ADH2 promoter may be grown for expression in a rich medium consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 mg/ml adenine and 80 mg/ml uracil. Derepression of the ADH2 promoter occurs upon exhaustion of medium glucose. Crude yeast supernatants are harvested by filtration and held at 4°C prior to further purification.

15                   Various mammalian or insect (*e.g.*, *Spodoptera* or *Trichoplusia*) cell culture systems can also be employed to express recombinant protein. Baculovirus systems for production of heterologous proteins in insect cells are reviewed, for example, by Luckow and Summers, *Bio/Technology* 6:47, 1988. Examples of suitable mammalian host cell lines include the COS-7 lines of monkey kidney cells, described  
20 by Gluzman (*Cell* 23:175, 1981), and other cell lines capable of expressing an appropriate vector including, for example, CV-1/EBNA (ATCC CRL 10478), L cells, C127, 3T3, Chinese hamster ovary (CHO), COS, NS-1, HeLa and BHK cell lines. Mammalian expression vectors may comprise nontranscribed elements such as an origin of replication, a suitable promoter and enhancer linked to the gene to be expressed, and  
25 other 5' or 3' flanking nontranscribed sequences, and 5' or 3' nontranslated sequences, such as necessary ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences.

                  Purified LeIF4A polypeptides may be prepared by culturing suitable host/vector systems to express the recombinant translation products of the DNAs of the  
30 present invention, which are then purified from culture media or cell extracts. For example, supernatants from systems which secrete recombinant protein into culture media may be first concentrated using a commercially available protein concentration filter, such as an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate may be applied to a suitable purification matrix. For  
35 example, a suitable affinity matrix may comprise a counter structure protein (*i.e.*, a protein to which LeIF4A binds in a specific interaction based on structure) or lectin or

antibody molecule bound to a suitable support. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step  
5 can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred. Gel filtration chromatography also provides a means of purifying LeIF4A.

Affinity chromatography is a particularly preferred method of purifying LeIF4A polypeptides. For example, a LeIF4A polypeptide expressed as a fusion  
10 protein comprising an immunoglobulin Fc region can be purified using Protein A or Protein G affinity chromatography. Moreover, a LeIF4A protein comprising a leucine zipper domain may be purified on a resin comprising an antibody specific to the leucine zipper domain. Monoclonal antibodies against the LeIF4A protein may also be useful in affinity chromatography purification, by utilizing methods that are well-known in the  
15 art.

Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media (*e.g.*, silica gel having pendant methyl or other aliphatic groups) can be employed to further purify a LeIF4A protein composition. Some or all of the foregoing purification steps, in  
20 various combinations, can also be employed to provide a homogeneous recombinant protein.

Recombinant LeIF4A polypeptide produced in bacterial culture is preferably isolated by initial extraction from cell pellets, followed by one or more concentration, salting-out, aqueous ion exchange or size exclusion chromatography  
25 steps. High performance liquid chromatography (HPLC) may be employed for final purification steps. Microbial cells employed in expression of recombinant LeIF4A protein can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Fermentation of yeast which express LeIF4A polypeptide as a secreted  
30 protein greatly simplifies purification. Secreted recombinant protein resulting from a large-scale fermentation can be purified by methods analogous to those disclosed by Urdal et al. (*J. Chromatog.* 296:171, 1984). This reference describes two sequential, reverse-phase HPLC steps for purification of recombinant human GM-CSF on a preparative HPLC column.

35 Preparations of LeIF4A polypeptides synthesized in recombinant culture may contain non-LeIF4A cell components, including proteins, in amounts and of a

character which depend upon the purification steps taken to recover the LeIF4A protein from the culture. These components ordinarily will be of yeast, prokaryotic or non-human eukaryotic origin. Such preparations are typically free of other proteins which may be normally associated with the LeIF4A protein as it is found in nature in its species of origin.

Automated synthesis provides an alternate method for preparing polypeptides of this invention having fewer than about 100 amino acids, and typically fewer than about 50 amino acids. For example, any of the commercially available solid-phase techniques may be employed, such as the Merrifield solid phase synthesis method, in which amino acids are sequentially added to a growing amino acid chain. (See Merrifield, *J. Am. Chem. Soc.* 85:2149-2146, 1963.) Equipment for automated synthesis of polypeptides is commercially available from suppliers such as Applied Biosystems, Inc. of Foster City, CA, and may generally be operated according to the manufacturer's instructions.

As an alternative to the presentation of LeIF4A polypeptides, the subject invention includes compositions capable of delivering nucleic acid molecules encoding an LeIF4A polypeptide or portion thereof. Such compositions include recombinant viral vectors (e.g., retroviruses (see WO 90/07936, WO 91/02805, WO 93/25234, WO 93/25698, and WO 94/03622), adenovirus (see Berkner, *Biotechniques* 6:616-627, 1988; Li et al., *Hum. Gene Ther.* 4:403-409, 1993; Vincent et al., *Nat. Genet.* 5:130-134, 1993; and Kolls et al., *Proc. Natl. Acad. Sci. USA* 91:215-219, 1994), pox virus (see U.S. Patent No. 4,769,330; U.S. Patent No. 5,017,487; and WO 89/01973)), naked DNA (see WO 90/11092), nucleic acid molecule complexed to a polycationic molecule (see WO 93/03709), and nucleic acid associated with liposomes (see Wang et al., *Proc. Natl. Acad. Sci. USA* 84:7851, 1987). In certain embodiments, the DNA may be linked to killed or inactivated adenovirus (see Curiel et al., *Hum. Gene Ther.* 3:147-154, 1992; Cotton et al., *Proc. Natl. Acad. Sci. USA* 89:6094, 1992). Other suitable compositions include DNA-ligand (see Wu et al., *J. Biol. Chem.* 264:16985-16987, 1989) and lipid-DNA combinations (see Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417, 1989). In addition, the efficiency of naked DNA uptake into cells may be increased by coating the DNA onto biodegradable latex beads.

In addition to direct *in vivo* procedures, *ex vivo* procedures may be used in which cells are removed from an animal, modified, and placed into the same or another animal. It will be evident that one can utilize any of the compositions noted above for introduction of LeIF4A nucleic acid molecules into tissue cells in an *ex vivo*

context. Protocols for viral, physical and chemical methods of uptake are well known in the art.

As noted above, the subject invention provides methods of using the polypeptides or related nucleic acid compositions disclosed herein for enhancing or eliciting immune responses. It has been found within the present invention that LeIF4A contains epitope(s) that stimulate proliferation of PBMCs from *Leishmania*-infected individuals. LbeIF4A also stimulates PBMCs from infected individuals to generate an exclusive Th1 cytokine profile. A Th1 response is characterized by the production of the cytokines interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-12 (IL-12) or interferon- $\gamma$  (IFN- $\gamma$ ), as well as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). IL-12 is a heterodimeric molecule comprising p40 and p35 subunits, which must be coexpressed for the production of biologically active IL-12 p70. The p40 subunit is produced only by IL-12-producing cells and is induced *in vitro* and *in vivo* after bacterial and parasite stimulation, whereas the p35 subunit is both ubiquitous and constitutively expressed. Therefore, cells producing IL-12 also have a large excess (10-100 fold) of biologically inactive free p40 chains. The stimulation of IL-12 production is particularly significant as this cytokine has the ability to influence T cells towards a Th1 response (IFN- $\gamma$  and IL-2 production). The ability of a protein to stimulate IL-12 production is therefore an important adjuvant property.

LeIF4A also stimulates a Th1 profile of mRNAs encoding IFN- $\gamma$ , IL-2, IL-12 p40 subunit, and TNF- $\alpha$ , in PBMCs from *Leishmania* infected patients. No detectable IL-4 or IL-10 mRNA, indicative of a Th2 response, is present in such stimulated PBMCs. In fact, LeIF4A generally down-regulates the expression of such Th2-associated cytokines. In addition, LeIF4A stimulates expression of IL-18 mRNA. These properties of LeIF4A suggest a role for LeIF4A in generating a protective or therapeutic immune response in leishmaniasis patients.

In addition, LeIF4A stimulates the production of IL-12 and IL-2 in PBMCs obtained from uninfected control individuals, as well as in cultured human macrophages, in the human myeloid leukemia cell line THP-1 and in mice. LeIF4A also synergizes with IFN- $\gamma$  to stimulate THP-1 cells to secrete IL-12, and the induction of IFN- $\gamma$  production by patient PBMCs is abrogated by the presence of anti-IL-12 antibody. The ability to stimulate IL-12 and IL-2 production indicates that LeIF4A has the ability to induce an immune response, and that the polypeptides described herein have a wide applicability in the non-specific enhancement of immune responses.

Accordingly, the present invention discloses methods for enhancing or eliciting, in a patient or cell culture, a cellular immune response (*e.g.*, the generation of

antigen-specific cytolytic T cells). The present invention also discloses methods for enhancing or eliciting a humoral immune response to an antigen (*e.g.*, antigen-reactive antibody production) using a LeIF4A polypeptide (*i.e.*, LbeIF4A, LmeIF4A or a variant thereof) as described above. As used herein, the term "patient" refers to any warm-blooded animal, preferably a human. A patient may be afflicted with a disease, such as leishmaniasis (or other infectious diseases) or cancer, such as melanoma, breast cancer, prostate cancer, lymphoma, colon cancer or other tumor, or may be normal (*i.e.*, free of detectable disease and infection). A patient may also, or alternatively, be afflicted with any Th2-mediated disease including, but not limited to, asthma, allergy, Th2-mediated autoimmune disease or Helminth infection. A "cell culture" is any preparation of PBMCs or isolated component cells (including, but not limited to, macrophages, monocytes, B cells and dendritic cells). Such cells may be isolated by any of a variety of techniques well known to those of ordinary skill in the art (such as Ficoll-hypaque density centrifugation). The cells may (but need not) have been isolated from a patient afflicted with leishmaniasis, or another disorder, and may be reintroduced into a patient after treatment.

Within these methods, the LeIF4A polypeptide (or nucleic acid composition) is administered to a patient or cell culture along with an antigen, such that it functions as an immunomodulating agent to enhance or elicit the immune response to the antigen. Within certain embodiments, one or more Th1-associated cytokines (*e.g.*, IL-2, IL-12, IL-15 and/or IL-18) may also be administered in combination with the LeIF4A polypeptide. The LeIF4A polypeptide may be administered within the same preparation (*e.g.*, vaccine) as the antigen, or may be administered separately. In one embodiment, the antigen and the LeIF4A polypeptide are administered to a patient at the same time and site. In this manner, LeIF4A polypeptides may be used, for example, as adjuvants in vaccine preparations for heterologous agents. In another embodiment, the antigen and LeIF4A polypeptide are administered at different sites on the patient. For example, the LeIF4A polypeptide could be administered (*e.g.*, injected) in one arm, and the antigen administered in the other arm. Such administrations may, but need not, take place at the same time. Alternatively, the LeIF4A polypeptide may be administered before or after the antigen. For example, the LeIF4A polypeptide could be administered 24 hours prior to antigen administration. Suitable doses and methods of administration are presented in detail below.

The immune response generated by a patient to whom a LeIF4A polypeptide is administered may vary, depending on the condition of the patient. For *Leishmania*-infected patients, the immune responses that may be generated include a

preferential Th1 immune response (which includes stimulation of IL-12 production) and the down-regulation of expression of Th2-associated cytokines, such as IL-4, IL-5 and/or IL-10. For uninfected individuals, the immune response may be the production of IL-12, the production of IL-2, the stimulation of gamma T cells, the production of interferon, the generation of antigen-reactive CTL, the production of antigen-specific antibodies or any combination thereof. Either type of response provides enhancement of the patient's immune response to the antigen administered with the LeIF4A polypeptide. In addition, for patients with diagnosed cancer, such as melanoma, breast cancer, lymphoma, colon cancer, prostate cancer and the like, the immune response may include a preferential CTL response. For treatment of a tumor, the immune response should result in a reduction in tumor mass.

The LeIF4A polypeptide (or nucleic acid composition) is preferably formulated for use in the above methods as a pharmaceutical composition or a vaccine. Pharmaceutical compositions generally comprise one or more LbeIF4A polypeptides in combination with a physiologically acceptable carrier, excipient or diluent. Such carriers will be nontoxic to recipients at the dosages and concentrations employed. The vaccines comprise one or more LbeIF4A polypeptides and one or more additional antigens appropriate for the indication. The use of LbeIF4A proteins in conjunction with soluble cytokine receptors, cytokines, and chemotherapeutic agents is also contemplated.

Routes and frequency of administration and polypeptide (or nucleic acid composition) doses will vary from individual to individual and may parallel those currently being used in immunization or treatment of other infections. In general, the pharmaceutical compositions and vaccines may be administered by injection (e.g., intramuscular, intravenous or subcutaneous), intranasally (e.g., by aspiration) or orally. The amount and frequency of administration will depend, of course, on such factors as the nature and severity of the indication being treated, the desired response, the condition of the patient, and so forth. Typically, between 1 and 4 doses may be administered for a 2-6 week period. Preferably, two doses are administered, with the second dose 2-4 weeks later than the first. A suitable dose is an amount of LeIF4A polypeptide that stimulates the production of IL-12 in the patient, such that the amount of IL-12 in supernatants of PBMCs isolated from the patient is between about 10 ng and 10 µg per mL. In general, the amount of IL-12 may be determined using any appropriate assay known to those of ordinary skill in the art, including the assays described herein. The amount of LbeIF4A polypeptide present in a dose typically ranges from about 1 pg to about 100 mg per kg of host, typically from about 10 pg to

about 1 mg, and preferably from about 100 pg to about 1 µg. Suitable dose sizes will vary with the size of the animal, but will typically range from about 0.01 mL to about 5 mL for 10-60 kg animal. Specific appropriate dosages for a particular indication can be readily determined.

5                   Alternatively, cells, preferably peripheral blood mononuclear cells, are removed from a patient and stimulated *in vitro* with one of the LeIF4A polypeptides and an antigen (including a tumor antigen). Upon generation of an antigen-specific immune response, such as a CTL response, the cells may be expanded and reinfused into the patient.

10                   While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will vary depending on the mode of administration and whether a sustained release administration is desired. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral  
15 administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable microspheres (*e.g.*, polylactic galactide) may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S.  
20 Patent Nos. 4,897,268 and 5,075,109 and in U.S. Patent Application Nos. 08/116,484 and 08/116,802 (incorporated by reference herein). The polypeptide or polypeptide/antigen combination may be encapsulated within the biodegradable microsphere or associated with the surface of the microsphere. In this regard, it is preferable that the microsphere be larger than approximately 25 microns.

25                   Pharmaceutical compositions and vaccines may also contain diluents such as buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrans, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with nonspecific serum albumin  
30 are exemplary appropriate diluents. Preferably, product is formulated as a lyophilizate using appropriate excipient solutions (*e.g.*, sucrose) as diluents.

                    Optionally, any of a variety of additional agents may be employed in the vaccines or pharmaceutical compositions of this invention, in addition to the LeIF4A polypeptide, to further nonspecifically enhance the immune response. Such agents  
35 usually contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a nonspecific stimulator of immune

responses, such as lipid A, *Bordetella pertussis* or *Mycobacterium tuberculosis*. Such agents are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, MI) and Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ).

5

The following examples are offered by way of illustration, and not by way of limitation. Those skilled in the art will recognize that variations of the invention embodied in the examples can be made, especially in light of the teachings of the various references cited herein.



## EXAMPLES

EXAMPLE 1Preparation of DNA Encoding LbeIF4A

5

This example illustrates the molecular cloning of a DNA sequence encoding the *L. braziliensis* ribosomal antigen LbeIF4A.

A genomic expression library was constructed with sheared DNA from *L. braziliensis* (MHOM/BR/75/M2903) in bacteriophage  $\lambda$ ZAPII (Stratagene, La Jolla, CA). The expression library was screened with *E. coli*-preadsorbed patient sera from an *L. braziliensis*-infected individual with mucosal leishmaniasis. Plaques containing immunoreactive recombinant antigens were purified, and the pBSK(-) phagemid excised using the manufacturer's protocols. Nested deletions were performed with Exonuclease III to generate overlapping deletions for single stranded template  
15 preparations and sequencing. Single stranded templates were isolated following infection with VCSM13 helper phage as recommended by the manufacturer (Stratagene, La Jolla, CA) and sequenced by the dideoxy chain terminator method or by the Taq dye terminator system using the Applied Biosystems Automated Sequencer Model 373A.

The immunoreactive recombinant antigens were then analyzed in patient  
20 T cell assays for their ability to stimulate a proliferative response, as described in Example 5, below, and a dominant Th1 cytokine profile, as described in Example 7, below.

A recombinant clone was identified in the above assays which, following sequence comparison of its predicted amino acid sequence with sequences of other  
25 proteins, was identified as a *Leishmania braziliensis* homologue of the eukaryotic initiation factor 4A (eIF4A). The isolated clone (pLeIF.1) lacked the first 48 amino acid residues (144 nucleotides) of the full length protein sequence. The pLeIF.1 insert was subsequently used to isolate the full length genomic sequence.

SEQ ID NO:1 shows the entire nucleotide sequence of the full-length  
30 LbeIF4A polypeptide. The open reading frame (nucleotides 115 to 1323) encodes a 403 amino acid protein with a predicted molecular weight of 45.3 kD. A comparison of the predicted protein sequence of LbeIF4A with the homologous proteins from tobacco (TeIF4A), mouse (MeIF4A), and yeast (YeIF4A) shows extensive sequence homology, with the first 20-30 amino acids being the most variable. The lengths (403, 413, 407,  
35 and 395 amino acids), molecular weights (45.3, 46.8, 46.4, and 44.7 kDa), and isoelectric points (5.9, 5.4, 5.5, and 4.9) of LbeIF4A, TeIF4A, MeIF4A and YeIF4A,

respectively, are similar. LbeIF4A shows an overall homology of 75.5% (57% identity, 18.5% conservative substitution) with TeIF4A, 68.6% (50% identity, 18.6% conservative substitution) with MeIF4A and 67.2% (47.6% identity, 19.6% conservative substitution) with YeIF4A.

5

## EXAMPLE 2

### Characterization of the LbeIF4A Gene

This example describes a Southern blot analysis of LbeIF4A DNA in  
10 *Leishmania* species. *Leishmania braziliensis* (MHOM/BR/75/M2903), *L. guyanensis* (MHOM/BR/75/M4147), *L. amazonensis* (IFLA/BR/67/PH8), *L. chagasi* (MHOM/BR/82/BA-2, Cl and MHOM/BR/84/Jonas), *L. donovani* (MHOM/Et/67/HU3), *L. infantum* (IPT-1), *L. major* (LTM p-2), *L. tropica* (1063C), *Trypanosoma cruzi* (MHOM/CH/00/Tulahuen C2) and *T. brucei* (TREU 667) were used and have been  
15 previously described (see, Burns et al., *Proc. Natl. Acad. Sci. U.S.A.* 90:775-779, 1993). Promastigotes and epimastigotes were cultured in axenic media. *L. chagasi* and *L. amazonensis* amastigotes were obtained from spleens of Syrian hamsters and footpads of BALB/c ByJ mice respectively, and purified as described in Burns et al., *J. Immunol.* 146:742-748, 1991.

20 Genomic DNA was prepared, digested with enzymes which cut both within (*Pst* I and *Not* I) and outside of LbeIF4A (*Bam*H I, *Eco*R I, *Eco*R V, *Hind* III, *Pvu* II, and *Sst* I), separated on 0.7% agarose gel and blotted onto Nytran (nylon) membrane, as described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, 1989. A restriction fragment  
25 comprising a ~0.94 kb fragment (nucleotides 143 to 1083) of the coding region of LbeIF4A was radiolabeled by the random priming method (see, Feinberg and Vogelstein, *Anal. Biochem.* 137:266-268, 1984) and blots were hybridized overnight at 65°C. Blots were washed twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1% SDS. *L. braziliensis* genomic DNA contained at least two copies  
30 of LbeIF4A as exemplified by the presence of two hybridizing bands in the *Bam*H I and *Pvu* II lanes (Fig. 1).

The same figure also illustrates the cross-species conservation between the eIF4A homologue of *L. braziliensis* and other *Leishmania* species. Two major *Pst* I hybridizing fragments were detected in all other *Leishmania* species tested with  
35 members of the *L. donovani* complex (*L. chagasi*, *L. donovani*, and *L. infantum*) showing identical hybridization patterns. LbeIF4A also cross-hybridizes with the more

distantly related parasite *T. cruzi* but not *T. brucei* under stringent hybridization conditions. These data show extensive cross-species conservation of the *Leishmania* eIF4A homologue.

5

### EXAMPLE 3

#### Preparation of LbeIF4A

This example illustrates the expression and purification of the ~45 kDa LbeIF4A antigen gene product. The 45 kDa recombinant antigen of the genomic clone pLeIF.1 (*i.e.*, the antigen lacking the N-terminal 48 residues) was purified from 500 ml of IPTG-induced cultures. The inclusion bodies were isolated and sequentially washed in 10 ml TNE (50 mM Tris, pH 8.0, 100 mM NaCl and 10 mM EDTA) containing 2, 4 and 8 M urea. Fractions containing solubilized recombinant antigen (usually the 4 and 8 M urea supernatants) were pooled, dialyzed against Tris-buffered saline (TBS) and concentrated by precipitation with 30% ammonium sulfate. Purification to homogeneity was accomplished by preparative SDS-PAGE electrophoresis, followed by excision and electroelution of the recombinant antigens. All antigens used in our studies had less than 10 pg/ml or 1 ng/mg protein endotoxin in a Limulus amebocyte assay performed by Immunex Corp., Seattle, WA. These amounts of endotoxin are insignificant for cytokine induction and/or adjuvant activity.

The recombinant antigen was used to immunize a rabbit for the production of a polyclonal anti-serum. An adult rabbit (New Zealand White; R & R Rabbitry, Stanwood, WA) was immunized by subcutaneous immunization with 100 µg of purified LbeIF4A in incomplete Freund's adjuvant (IFA, GIBCO, Grand Island, NY) together with 100 µg of muramyl dipeptide (adjuvant peptide, Calbiochem-Novabiochem Corp., La Jolla, CA), followed by a boost four weeks later with 100 µg of the recombinant antigen in IFA alone. Three weeks later, the rabbit was boosted intravenously with 25 µg of LbeIF4A in saline and serum was collected one week later.

Immunoblots of *L. braziliensis* lysates from promastigotes harvested during the early-, mid-, or late-log phases or following a temperature shift of the culture from 22-35°C were subsequently performed with the polyclonal rabbit anti-serum as a probe (Fig. 2). Panel A of Figure 2 shows the immunoblot analysis of molecular weight markers (lane M), *E. coli* lysates from uninduced (lane 1) and induced (lane 2) cultures, and the purified recombinant antigen (lane 3). Panel B of Figure 2 shows the immunoblot analysis of *L. braziliensis* promastigote lysate (lane 1), *L. chagasi*

promastigote lysate (lane 2), and *L. amazonensis* promastigote (lane 3) or amastigote (lane 4) lysate.

Parasite and mammalian cell lysates were prepared by freeze/thaw lysis of pellets in SDS sample buffer without glycerol and  $\beta$ -mercaptoethanol. Insoluble material was separated from the supernatant by centrifugation at 10K rpm in a microfuge. Protein concentrations were determined using the Pierce BCA protein assay kit. Five to 10  $\mu$ g of parasite or cell extracts or 0.5 to 1.0  $\mu$ g of recombinant antigens were separated on 12.5% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. Reactivities of the antisera were assessed as previously described (Skeiky et al., *J. Exp. Med.* 176:201-211, 1992) using [ $^{125}$ I]-Protein A, followed by autoradiography.

The rabbit anti-serum detected one dominant protein species of size ~45 kD. The relative intensities of the 45 kD eIF4A homologue were similar for all the lysates analyzed, thus suggesting that this antigen is constitutively expressed during the early- to mid-log growth phase of the parasite or following a temperature transition that mimics the intracellular amastigote stage. This is unlike members of the *Leishmania* heat-shock protein family whose products are upregulated following a temperature transition from 22-35 °C. The pre-immune rabbit serum did not react with the parasite lysates.

20

#### EXAMPLE 4

##### Preparation of Monoclonal Antibodies that Bind to LbeIF4A

This example illustrates the preparation of monoclonal antibodies against LbeIF4A. Preparations of purified recombinant LbeIF4A or transfected cells expressing high levels of LbeIF4A, may be employed to generate monoclonal antibodies against LbeIF4A using conventional techniques, such as those disclosed in U.S. Patent No. 4,411,993. Such antibodies may be used to interfere with LbeIF4A activation of PBMCs, as components of diagnostic or research assays for LbeIF4A, or in affinity purification of LbeIF4A.

To immunize rodents, LbeIF4A immunogen is emulsified in an adjuvant (such as complete or incomplete Freund's adjuvant, alum, or another adjuvant, such as Ribi adjuvant R700 (Ribi, Hamilton, MT), and injected in amounts ranging from 10-100  $\mu$ g subcutaneously into a selected rodent, for example, BALB/c mice or Lewis rats. Ten days to three weeks days later, the immunized animals are boosted with additional immunogen and periodically boosted thereafter on a weekly, biweekly or every third

week immunization schedule. Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision for testing by dot-blot assay (antibody sandwich) or ELISA (enzyme-linked immunosorbent assay). Other assay procedures are also suitable, such as inhibition of the elicitation of a Th1 response.

5           Following detection of an appropriate antibody titer, positive animals are given an intravenous injection of antigen in saline. Three to four days later, the animals are sacrificed, splenocytes harvested, and fused to a murine myeloma cell line (e.g., NS1 or preferably Ag 8.653 [ATCC CRL 1580]). Hybridoma cell lines generated by this procedure are plated in multiple microtiter plates in a selective medium (for  
10           example, one containing hypoxanthine, aminopterin, and thymidine, or HAT) to inhibit proliferation of non-fused cells, myeloma-myeloma hybrids, and splenocyte-splenocyte hybrids.

          Hybridoma lines thus generated can be screened by ELISA for reactivity with LbeIF4A, for example, by adaptations of the techniques disclosed by Engvall et al.  
15           (*Immunochem.* 8:871, 1971) and in U.S. Patent No. 4,703,004. A preferred screening technique is the antibody capture technique described by Beckman et al., *J. Immunol.* 144:4212 (1990). The hybridoma lines are cloned, for example, by limiting dilution or by cloning in soft agar, to yield a monoclonal cell line. Positive clones are then injected into the peritoneal cavities of syngeneic rodents to produce ascites containing high  
20           concentrations (>1 mg/ml) of anti-LbeIF4A monoclonal antibody. The resulting monoclonal antibody can be purified by ammonium sulfate precipitation followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to protein A or protein G can also be used, as can affinity chromatography based upon binding to LbeIF4A.

25

#### EXAMPLE 5

##### LbeIF4A Stimulation of PBMC Proliferation

          This example illustrates the ability of purified recombinant LbeIF4A to  
30           stimulate proliferation of PBMCs from *L. braziliensis*-infected individuals. Peripheral blood was obtained from individuals living in an area (Corte de Pedra, Bahia, Brazil) endemic to *L. braziliensis* transmission where epidemiological, clinical, and immunological studies have been performed for over a decade. Diagnosis of the patients was made by clinical findings associated with at least one of the following:  
35           isolation of parasite from lesions, a positive skin test with *Leishmania* lysate or a positive serological test.

Peripheral blood was collected and PBMCs isolated by density centrifugation through Ficoll™ (Winthrop Laboratories, New York). For *in vitro* proliferation assays, 2 - 4 x 10<sup>5</sup> cells/well were cultured in complete medium (RPMI 1640 supplemented with gentamycin, 2-ME, L-glutamine, and 10% screened pooled A+ human serum; Trimar, Hollywood, CA) in 96-well flat bottom plates with or without 10 µg/ml of the indicated antigens or 5 µg/ml PHA (Sigma Immunochemicals, St. Louis, MO) for five days. The cells were then pulsed with 1 µCi of [<sup>3</sup>H] thymidine for the final 18 hours of culture.

Data are represented as mean cpm of triplicate cultures and the stimulation index (SI) defined as mean cpm of cultures with antigens/mean cpm of cultures without antigen. As shown in Table I and Figure 3, PBMCs from most (>70%) mucosal and active or healed cutaneous patients responded to LbeIF4A with a heterogeneous proliferation pattern with stimulation indices ranging from 12 to 233 and 2 to 64 respectively.

**TABLE I**  
**In Vitro Proliferation of PBMCs from *L. braziliensis*-infected Individuals**  
**in Response to Parasite Lysate and LbeIF4A Antigens**

5	PATIENTS	<u>[<sup>3</sup>H]Tdr Incorporation (Mean cpm (SD) x 10<sup>-3</sup>)</u>					
		MEDIA		LYSATE		LbeIF4A	
				S.I.		S.I.	
	MUCOSAL						
	JV	0.15	(0.0)	41.30(1.3)	294	11.90(4.8)	81
10	SZ	0.45	(0.1)	140.60(7.6)	308	105.90(5.6)	233
	AB	0.42	(0.3)	44.20(0.5)	104	5.00(1.3)	12
	NO	0.38	(0.1)	52.70(3.3)	138	12.80(1.6)	33
	TE	0.18	(0.0)	27.40(1.5)	150	8.80(0.3)	48
	MB	0.18	(0.0)	300.10(9.4)	1634	41.50(4.5)	226
15	OM	0.28	(0.0)	35.40(3.2)	124	6.90(2.5)	24
	CUTANEOUS						
	AS	0.22	(0.0)	19.14(1.3)	87	14.30(2.3)	64
	JP	0.25	(0.0)	55.63(8.6)	218	4.40(0.3)	17
20	VS	0.17	(0.0)	0.26(0.0)	1.5	0.3 (0.0)	2
	RJ	0.10	(0.0)	0.32(0.2)	3.0	1.5 (0.6)	15
	JA	0.16	(0.0)	0.77(0.1)	4.7	2.5 (0.2)	16
	AD	4.20	(1.0)	4.01(1.0)	0.0	14.1 (2.2)	3.5
	HN	0.36	(0.0)	4.73(1.7)	13	4.69(1.7)	13
25	DIFFUSE CUTANEOUS						
	VAL	0.22	(0.0)	0.51(0.3)	2.0	2.12(0.2)	9.0
	SELF-HEALING CUTANEOUS						
	GS	0.21	(0.0)	19.70(4.4)	94	41.50(2.8)	198
30	MS	0.09	(0.0)	0.60(0.1)	6.5	5.10(2.1)	57
	AH	0.11	(0.0)	59.60(7.1)	519	9.60(4.7)	83
	DJ	0.12	(0.0)	0.20(0.1)	1.6	19.00(6.7)	151
	HS	0.12	(0.0)	27.10(2.0)	225	12.40(2.7)	103
	MCT	0.38	(0.0)	130.30 (14)	340	6.20(1.5)	16
35	NORMAL						
	LV	0.14	(0.0)	0.19(0.0)	1.4	0.71(0.1)	4.0
	VV	0.18	(0.0)	0.31(0.1)	1.7	0.28(0.1)	1.5
	N3	0.14	(0.0)	0.36(0.1)	2.6	0.27(0.1)	1.9
40	N4	0.59	(0.1)	2.00(0.3)	3.8	0.56(0.0)	1.0

In general, the stimulation indices were higher with PBMCs from mucosal individuals. PBMCs from some mucosal patients responded to LbeIF4A with stimulation indices comparable to those observed with parasite lysate. Interestingly, in some patients with cutaneous leishmaniasis, the proliferative responses to LbeIF4A were higher than those elicited by parasite lysate. In contrast to mucosal and cutaneous patients, PBMCs from all six individuals with self healing cutaneous leishmaniasis proliferated in response to LbeIF4A with stimulation indices (16-198) comparable to those of mucosal individuals. PBMCs from two of the self healing individuals (MS and DJ), had responses that were significantly higher than those obtained with parasite lysate. Cells from normal uninfected individuals were only marginally stimulated by LbeIF4A.

#### EXAMPLE 6

##### LbeIF4A Stimulation of Cytokine mRNA Expression in PBMCs

This example presents an analysis of cytokine mRNA expression patterns of PBMCs from patients with confirmed cases of *L. braziliensis* infection. For cytokine mRNA analysis, 0.5 to 1 ml of PBMCs were cultured at  $1-2 \times 10^6$  cells/ml with or without 10  $\mu\text{g/ml}$  of the LbeIF4A antigen lacking the N-terminal 48 residues of SEQ ID NO:2 (as described in Example 3) for 48 and 72 hours. The supernatants and cells were harvested and analyzed for cytokine mRNAs by polymerase chain reaction (PCR). For cytokine mRNA PCR analysis, total RNA was isolated from the PBMCs using the acid guanidium thiocyanate-phenol-chloroform extraction method, as described by Chomczynski and Sacchi (*Anal. Biochem.* 162:156-159, 1987). Complementary DNA (cDNA) was synthesized using poly(dT) (Pharmacia) and AMV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) in a final volume of 20  $\mu\text{l}$ . cDNA samples were brought to 200  $\mu\text{l}$  with water.

Following normalization to  $\beta$ -actin, 12 to 20  $\mu\text{l}$  of diluted cDNA were amplified by PCR using Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) with 0.2  $\mu\text{M}$  of the respective 5' and 3' external primers in a reaction volume of 50  $\mu\text{l}$ . The conditions used were: denaturation at 94°C (1 minute for  $\beta$ -actin, IL-2, and IL-4; 45 sec for IFN- $\gamma$  and 30 sec for IL-10), annealing at 55°C (1 minute for  $\beta$ -actin, IL-2, and IL-4; 30 sec for IL-10) or 60°C for 45 sec for IFN- $\gamma$  and elongation at 72°C. We verified that our PCR conditions were within the semi-quantitative range by initially performing serial dilutions of the cDNAs and varying the number of cycles used for PCR. In all



subsequent experiments, 30 cycles were used in the amplification reactions for  $\beta$ -actin, IL-2, IL-4, and IFN- $\gamma$ . In the case of IL-10 PCR, 25 cycles were used.

The primer pairs used and the PCR conditions were from published information;  $\beta$ -actin, IL-2, IL-4 and IFN- $\gamma$  (Ehlers et al., *J. Exp. Med.* 173:23-36, 1991) and IL-10 (Viera et al., *Proc. Natl. Acad. Sci. U.S.A.* 88:1172-1176, 1991). The nucleotide sequences for the 5' and 3' oligonucleotide primers, respectively, were as follows: (1)  $\beta$ -actin, TGACGGGGTCACCCACACTGTGCCCATCTA and CTAGAAGCATTGCGGTGGACGATGGAGGG; (2) IL-2, ATGTACAGGATGCA ACTCCTGTCTT and GTCAGTGTTGAGATGATGCTTTGAC; (3) IL-4, 10 ATGGGTCTCACCTCCCAACTGCT and CGAACACTTTGAATATTTCTCT CTCAT; (4) IFN- $\gamma$ , ATGAAATATACAAGTTATATCTTGGCTTT and GATGCTCTTCGACCTCGAAACAGCAT; (5) IL-10, TCTCAAGGGGCTGG GTCAGCTATCCCA and ATGCCCCAAGCTGAGAACCAAGACCCA.

Probes were obtained using plasmids containing the human sequences 15 IL-2, IFN- $\gamma$  and IL-4 (Lewis et al., *Proc. Natl. Acad. Sci. U.S.A.* 85:9743-9747, 1988) and  $\beta$ -actin (no. 65128; American Type Culture Collection, Rockville, MD), which were digested with *Hind* III/*Eco* R I, *Eco* R I, *Sac* I/*Hind* III, and *Eco* R I respectively. Human IL-10 cDNA was cloned by PCR from mitogen-stimulated PBMCs from normal donors using oligonucleotide primers designed to amplify a 535 base pair fragment 20 spanning the entire coding region of human IL-10 (Lewis et al., *Proc. Natl. Acad. Sci. U.S.A.* 85:9743-9747, 1988). The cDNA was subcloned into pBluescript and digested with *Bam* H I/*Eco* R I. After separation on 1% agarose gels, insert DNA fragments were excised, electroeluted, and purified. Radiolabeled  $^{32}$ P-probes were prepared by the random priming method.

25 PCR products were analyzed by electrophoresis on 1.5% agarose gels, transferred to nylon membranes, and probed with the appropriate  $^{32}$ P-labeled DNA insert. Hybridizations were at 55°C overnight. Post hybridization washes were at 55°C for 20 minutes twice each with 2x, and 1x SSC containing 0.2% SDS.

The results of these analyses are presented in Figures 4A and 4B. PCR 30 cytokine analyses were performed with cells prior to culturing (lanes 0), following culturing in the absence of antigen (lanes -), or following culturing in the presence of 10  $\mu$ g/ml *L. braziliensis* lysate (lanes Lb) or in the presence of 10  $\mu$ g/ml LbIF4A (lanes IF). Fig. 4A shows the PCR results of cytokine mRNA for three of the six mucosal patients' PBMCs analyzed (JV, SZ, and TE) and one patient (VA) with *L. amazonensis* 35 infection, manifested as diffuse cutaneous leishmaniasis (DCL). In three of the six mucosal patients (TE, Fig. 4A; NO and EO, not shown), PBMCs not cultured *in vitro*

had detectable levels of mRNA for IFN- $\gamma$  and IL-4, as well as IL-2 (patients TE and EO). IL-10 mRNA was not detected in the "resting" PBMCs from any of the mucosal patients. However, following *in vitro* culturing in the absence of antigen stimulation, the synthesis of IL-10 mRNA was upregulated in most of the mucosal PBMCs analyzed. In addition, the levels of cytokine mRNAs detected in the "resting" PBMCs of patients TE, NO, and EO, decreased to background levels.

Parasite lysate stimulated the expression of mRNAs of the Th1 cytokines IFN- $\gamma$  and IL-2 as well as that of the Th2 cytokine IL-4 (in three of the six patients). Increased IL-10 mRNA was detected in one of the patients' PBMCs (SZ) following culture with the parasite lysate. Both LbeIF4A antigen and parasite lysate elicited the production of mRNA of IFN- $\gamma$  and IL-2 from all mucosal patient PBMCs with LbeIF4A eliciting an exclusive Th1 cytokine profile. In fact, LbeIF4A downregulated the synthesis of IL-10 mRNA detected in the cultured PBMCs of most mucosal patients prior to antigen stimulation. Interestingly, as with the case of using PBMCs from mucosal patients, LbeIF4A also downregulated the synthesis of IL-10 mRNA in the DCL patient VA.

In general, the levels of mRNAs for IFN- $\gamma$  and IL-2 increased from undetectable amounts prior to antigen stimulation to readily visual levels following antigen stimulation in ethidium bromide stained gels. However, mRNA for the cytokines IL-4 and IL-10, were only detected following radioactive probing of the resolved PCR products, indicating low abundance of these cytokine messages.

Similar PCR analysis was performed on PBMCs derived from cutaneous patients (Fig. 4B). The fresh PBMCs from three (VS, JP and CA (not shown)) of the four patients analyzed revealed high levels of mRNAs for both the Th1 (IFN- $\gamma$  and IL-2) and Th2 (IL-4 and IL-10) cytokines examined. mRNAs for IFN- $\gamma$  and IL-2, but not for IL-10 and IL-4, were detected in the fresh PBMCs of the fourth (AS) cutaneous patient. Therefore, in contrast to mucosal patients, patients with cutaneous leishmaniasis have IL-10 mRNA, in addition to IL-4, IL-2, and IFN- $\gamma$ , in their fresh PBMCs. Interestingly, while the mRNAs for IL-2 and IFN- $\gamma$  were reduced to barely detectable levels following the *in vitro* culturing of PBMCs in the absence of antigen, those for IL-10 remained either unaffected or increased. Therefore, in cutaneous patients, the spontaneous levels of IL-10 mRNA is either stable or their PBMCs continue to synthesize IL-10 mRNA in the absence of antigen stimulation. The observation of such a response for cutaneous leishmaniasis patients can be exploited to differentiate individuals who are predisposed to developing chronic cutaneous leishmaniasis from those who will experience self healing lesions.

All cutaneous patients tested responded to LbeIF4A antigen as well as to the parasite lysate by upregulating the synthesis of mRNAs for IL-2 and IFN- $\gamma$  and, in two of four patients (VS and AS), the level of IL-4 mRNA also increased following stimulation with parasite lysate. In the three patients (VS, JP and CA) with detectable spontaneous levels of IL-10 mRNA, LbeIF4A as well as the parasite lysate down-regulated the expression of IL-10, mRNA.

The cytokine mRNA profiles of PBMCs from patients with self-healing CL were similar to those of ML patients in that (a) except for one individual with detectable levels of IL-10 mRNA, fresh PBMCs from three of four patients analyzed had detectable levels of IL-2, IFN- $\gamma$  and IL-4, but little or no IL-10 mRNA; (b) IL-10 mRNA was upregulated after culture of PBMCs without antigen, whereas those of IL-2, IFN- $\gamma$  and IL-4 decreased to background levels; and (c) leishmanial lysate stimulated the expression of a mixed Th1/Th2 cytokine profile, whereas LbeIF4A elicited increased mRNA expression of only the Th1-type cytokines and downregulated the expression of IL-10 mRNA in the cultured PBMCs of most self-healing individuals (not shown).

#### EXAMPLE 7

##### LbeIF4A Stimulation of Cytokine Secretion in PBMCs

20

This example presents the supernatant levels of secreted cytokines of PBMCs from *L. braziliensis*-infected individuals following stimulation with LbeIF4A antigen lacking the N-terminal 48 residues of SEQ ID NO:2 (as described in Example 3) or parasite lysate. Aliquots of the PBMC supernatants were assayed for IFN- $\gamma$ , TNF- $\alpha$ , IL-4, and IL-10. IFN- $\gamma$  was quantitated by a double sandwich ELISA using mouse anti-human IFN- $\gamma$  mAb (Chemicon, Temucula, CA) and polyclonal rabbit anti-human IFN- $\gamma$  serum. Human rIFN- $\gamma$  (Genentech Inc., San Francisco, CA) was used to generate a standard curve. IL-4 was quantitated in supernatants by a double sandwich ELISA using a mouse anti-human IL-4 mAb (M1) and a polyclonal rabbit anti-human IL-4 sera (P3). Human IL-4 (Immunex Corp., Seattle, WA) was used to generate a standard curve ranging from 50 pg/ml to 1 ng/ml. IL-10 was measured using a rat anti-human IL-10 mAb (PharMingen, San Diego, CA, Cat. # 18551D) to "capture" secreted IL-10 and a biotinylated rat antihuman IL-10 mAb (PharMingen San Diego, CA, Cat. # 18562D) for detection of bound IL-10 with streptavidin conjugated horse radish peroxidase and ABTS as substrate. A standard curve was obtained using human

rIL-10 (kindly provided by DNAX Research Institute, Palo Alto, CA), ranging from 30 pg to 2 ng/ml.

Cells from all three patient groups (*i.e.*, mucosal, cutaneous and self-healing cutaneous) secreted IFN- $\gamma$  and TNF- $\alpha$  following stimulation with either 10  $\mu$ g/ml LbeIF4A antigen or 10  $\mu$ g/ml parasite lysate (Figs. 5 and 6). Similarly, LbeIF4A stimulated PBMCs from patients with *L. tropica* infection (Desert Storm Patients) to proliferate and secrete IFN- $\gamma$  (not shown). The levels of both IFN- $\gamma$  and TNF- $\alpha$  detected in the supernatants of patient PBMCs were significantly higher than those from uninfected controls. In the absence of antigen stimulation, only PBMCs from mucosal patients (five of six) produced detectable levels of supernatant TNF- $\alpha$  (60 to 190 pg/ml). Little or no IL-4 or IL-10 was detected in any of the supernatants analyzed (not shown), indicating levels below the detection limit of the ELISA assay employed. By comparison, leishmanial lysate also stimulated PBMCs to secrete IFN- $\gamma$  and TNF- $\alpha$  and, in some patients, IL-10 was also detected (not shown). Taken together, the results demonstrate that LbeIF4A stimulated a predominant Th1 cytokine profile in PBMCs from *L. braziliensis*-infected individuals, whereas parasite lysate stimulated a mixed Th1/Th2 cytokine profile.

The levels of TNF- $\alpha$  detected in the supernatants of patient PBMCs from mucosal and self-healing individuals following antigen stimulation were higher than those from cutaneous patients (Fig. 6). PBMCs from four of five mucosal patients (JV, SZ, AB, and MB) had supernatant levels of TNF- $\alpha$  (0.80 to 2.20 ng/ml) higher than those detected in cultures of PBMCs from uninfected controls following stimulation with parasite lysate. Similarly, the same PBMCs were stimulated by LbeIF4A to produce supernatant levels of TNF- $\alpha$  with values ranging from 0.66 to 3.14 ng/ml. Compared to uninfected controls, PBMCs from three (GS, HS, and MCT) out of six self-healing individuals analyzed produced higher levels of TNF- $\alpha$  in response to parasite lysate, and all six (GS, MS, AH, DJ, HS, and MCT) out of six self-healing individuals analyzed produced higher levels of TNF- $\alpha$  in response to LbeIF4A. The levels of TNF- $\alpha$  produced by PBMCs from cutaneous leishmaniasis patients in response to parasite lysate were comparable to uninfected controls. However, LbeIF4A stimulated PBMCs in three of these patients (RJ, AD and JS) to produce TNF- $\alpha$ . Such patients may be in the process of developing acute cutaneous leishmaniasis.

### EXAMPLE 8

#### Stimulation of IL-12 Production by LbeIF4A

This example shows that LbeIF4A stimulates PBMCs from  
5 *L. braziliensis*-infected individuals, as well as PBMCs or cultured human macrophages, adherent PBMCs, from the blood of normal donors and the human myeloid leukemia cell-line THP-1, to secrete IL-12. IL-12 has been shown to play a pivotal immunoregulatory role in the development of cell mediated immunity, generation of Th1 responses and IFN- $\gamma$  production in intracellular bacterial or parasitic infections.  
10 The LbeIF4A polypeptide used was the LbeIF4A antigen lacking the N-terminal 48 residues of SEQ ID NO:2 (as described in Example 3).

IL-12 p40 was measured in cell-free supernatants by RIA (detection limit of 10 pg/ml) using the mAb pairs C11.79/C8.6, as described by D'Andrea et al. (*J. Exp. Med.* 179:1387-1398, 1992). Biologically active IL-12 p70 heterodimer (detection limit  
15 1 pg/ml) was measured as described by Kubin et al. (*Blood* 83:1847-1855, 1994).

Fig. 7A shows that 10  $\mu$ g/ml LbeIF4A (LeIF) stimulated mucosal patient PBMCs to secrete IL-12 p40 in the cultured supernatant with a magnitude significantly higher than the IL-12 p40 levels observed with 10  $\mu$ g/ml parasite lysate as antigen (Lb). The amount of IL-12 p40 secreted in the absence of lysate or antigen is also shown  
20 (Med). The same figure also shows that 10  $\mu$ g/ml IL-10 down-regulated the production of IL-12 p40 by patient PBMCs following stimulation with LbeIF4A (LeIF + IL-10) or lysate (Lb + IL-10).

PBMCs from uninfected individuals also produced IL-12 p40 when cultured with LbeIF4A (LeIF, Fig. 7B), although no p40 was detected in response to  
25 parasite lysate (Lb). This may suggest a role for IFN- $\gamma$  in the lysate-induced p40 observed in patient PBMCs, which produced 5-100 fold more IFN- $\gamma$  than normal PBMCs after antigen stimulation (see Fig. 5).

To determine whether the IL-12 p40 observed in antigen-stimulated PBMC cultures reflected biologically active cytokine, IL-12 p70 was also assayed in  
30 these cultures (Figs. 7C and 7D). In general, the p70 production paralleled that of p40, demonstrating that biologically active IL-12 was produced in response to LbeIF4A in both patient and normal PBMCs.

LbeIF4A also stimulates IL-12 production in cultured human macrophages (Fig. 9A) and in adherent PBMCs (Fig. 9B). Adherent cells were  
35 prepared from PBMCs separated by Ficoll-hypaque gradient centrifugation from the blood of normal donors.  $2 \times 10^6$  PBMCs were cultivated for 2 hours in 500 $\mu$ l RPMI,

2% human AB serum. Adherent cells were purified by washing the plates 3 times with PBS. Then 500µl of test medium (RPMI, 2% human AB serum) with the respective stimulus were added (IFN-1000U/ml, LbeIF4A (Lf) 10µg/ml). Supernatants were taken after 18 hours.

5 IL-12 production of adherent PBMCs was measured by a capture bio-assay with 5 day old PHA blast. Briefly, the IL-12 capture antibody C11.5.14 (kind gift of the Wistar Institute) was coated on 96 well plates. Supernatants of the induction experiment and recombinant IL-12, as a standard, were incubated for 4 hours. After several wash steps, 5 day old PHA blasts were added and the proliferation of these  
10 blasts was used to determine IL-12 concentrations in supernatants of adherent cells.

Macrophages were generated by cultivating adherent cells ( $2 \times 10^6$  PBMCs) for 5 days in test medium. Then, the macrophages were washed in PBS and 500 µl RPMI, 2% human AB serum, and 1000U/mL IFN-γ was added. Macrophages were stimulated with LbeIF4A (10 µg/ml) or cultivated in medium (M) alone. In one  
15 set, LbeIF4A control macrophages were incubated with LbeIF4A in 500 µl RPMI, 2% human AB serum, without IFN-γ. Supernatants were taken after 18 hours and used for induction of IL-12 dependent proliferation. Briefly, 5 day old blasts were incubated with macrophage supernatants for 2 days. For the last 18 hours,  $^3\text{H}$  thymidine was added. Neutralizing anti-IL-12 polyclonal goat serum (5 µg/ml) was added as indicated.

20 In addition, LbeIF4A stimulates IL-12 p40 production in the human myeloid leukemia cell-line, THP-1 (Fig. 10). The cells were cultured at  $10^6$  cells/ml for 24-48 hours in Endotoxin-free RPMI medium containing 5% Fetal Bovine serum. 10 µg/ml LbeIF4A synergized with IFN-γ to stimulate THP-1 cells to secrete IL-12 p40. These results indicate the utility of LbeIF4A as vaccine adjuvant.

25

### EXAMPLE 9

#### Effect of IL-12 and IL-10 on LbeIF4A Induction of IFN-γ Production

This Example examines the interaction among IL-12, IL-10 and IFN-γ in  
30 response to the LbeIF4A polypeptide lacking the N-terminal 48 residues of SEQ ID NO:2 (as described in Example 3). As shown in Fig. 8A, PBMCs from patients with mucosal leishmaniasis were stimulated with 10µg/ml LbeIF4A in the absence (LeIF) or presence of 10 ng/ml anti-IL-12 (LeIF + Anti-IL-12), or IL-10 (LeIF + IL-10), and the cultured supernatants were assayed for IFN-γ secretion. Both anti-IL-12 mAb and IL-  
35 10 abrogated the production of LbeIF4A-induced IFN-γ secretion. However, anti-IL-12 mAb only partially decreased the production of IFN-γ after stimulation with leishmanial

lysate (Fig. 8B). These results show that IFN- $\gamma$  production is IL-12 dependent, and is inhibited by IL-10, whereas the production of IL-12 is regulated by both IFN- $\gamma$  dependent and independent pathways.

5

#### EXAMPLE 10

##### LbeIF4A Stimulation of a TH1 Profile in Mice

This example demonstrates that the LbeIF4A polypeptide lacking the N-terminal 48 residues of SEQ ID NO:2 (as described in Example 3) stimulates a dominant Th1 cytokine profile in BALB/c mice. The animals were primed with either LbeIF4A or 8E (the C-terminal portion of the *L. braziliensis* mitochondrial hsp70, which stimulates patient PBMCs to produce high levels of IL-10) using quilA or CFA as adjuvants. Ten days after priming, lymph node (LN) cells were restimulated *in vitro* with the recombinant antigens and the supernatant cultures were analyzed for secreted cytokines. The results (Fig. 11) show that LN cells of mice primed with LbeIF4A proliferated and secreted an almost exclusive Th1 cytokine (IFN- $\gamma$ ) following challenge with LbeIF4A using both types of adjuvants. In contrast, LN cells from mice primed with 8E produced a Th0 response or Th1/Th2 type cytokine (with quilA as adjuvant) with a strong bias towards the Th2 cytokines, IL-4, and IL-10 in specific response to challenge with 8E. Similarly, mice primed with parasite lysate produced a mixed cytokine profile, a result that may argue against the use of parasite lysate alone as vaccine candidate (Fig. 11).

These results indicate that LbeIF4A may be used as an adjuvant, as well as a specific T cell vaccine. Because LbeIF4A induced a powerful Th1 response, including the two cytokines most clearly associated with protection in experimental leishmaniasis, IFN- $\gamma$  and IL-12, we studied the ability of this antigen to protect mice against leishmaniasis. BALB/c mice were immunized once with LbeIF4A with no adjuvant, followed by subcutaneous infection with *L. major* seven days later. Compared to the control group, LbeIF4A provided significant protection against *L. major* infection (Fig. 12). Thus a heterologous antigen derived from *L. braziliensis* can confer some protection to *L. major* infection, suggesting that, at least some of the "protective" epitopes are conserved between the two parasites.

EXAMPLE 11  
Preparation of LmeIF4A

This example illustrates the appropriation of new variant of LbeIF4A  
5 from *Leishmania major*.

A cDNA expression library was constructed with polyA<sup>+</sup> RNA of  
*L. major* using the ZAP-cDNA unidirectional cloning kit (Stratagene, La Jolla, CA).  
Approximately 500,000 pfu was screened with a radio-labeled DNA probe comprising  
nucleotides 258 to 1188 of SEQ ID NO. 1. Post-hybridization washes were at 55°C  
10 with 0.5x SSC containing 0.1% SDS. This resulted in the identification of a clone  
containing an approximately 2.5 kb insert (LmeIF4A). Excision of the pBSK (-)  
phagemid sequences was carried out according to the manufacturer's protocols.  
Overlapping clones were generated from the cDNA insert by exonuclease III and  
sequenced by the *Taq* dye terminator.

15 The approximately 2.7 kb cDNA insert of LmeIF4A was found to  
contain the entire coding sequence, as well as the 5' spliced leader and 3' flanking  
sequences. A fragment containing the entire coding sequence of LmeIF4A was  
amplified by PCR using 5' and 3' specific oligonucleotides. The 5' oligonucleotide  
contained an *Nde* I restriction site preceding the ATG initiation site. When cloned into  
20 the *Nde* I site of the pET vector (Novagen, Madison, WI), the first amino acid of the  
protein was the initiation code (Met) of the non-fusion recombinant LmeIF4A. Non-  
fusion full-length rLmeIF4A was produced in *E. coli*, using the pET plasma vector and  
a T7 polymerase expression system (Novagen, Madison, WI). The inclusion bodies  
were isolated and sequentially washed twice in 10 ml of TNE (50 mM TRIS, pH 8.0,  
25 100 mM NaCl, 10 mM EDTA) containing 2, 4, and 8 M urea. The 8 M urea fraction  
containing a recombinant antigen was applied to a preparative SDS-PAGE gel.  
Recombinant LmeIF4A was purified either by electroelution from the preparative gel or  
by HPLC. The final product had negligible levels of bacterial endotoxin, as determined  
by Limulus assay.

30 Partial sequence analysis of the LmeIF4A cDNA indicated substantial  
homology (at the nucleotide and amino acid level) (SEQ ID NOS:3 and 4) to the antigen  
isolated from *L. braziliensis* (i.e., greater than 90% amino acid sequence identity).



### EXAMPLE 12

#### Elicitation of Antigen-Specific CTL Response

This example illustrates the elicitation of a specific CTL response against  
5 soluble ovalbumin using LmeIF4A, prepared as described above.

C57BL/6 mice (H-2<sup>b</sup>) were immunized once with 30 µg of soluble  
ovalbumin (Sigma, St. Louis, MO), either alone (ova alone) or in combination with 50  
µg of LmeIF4A encapsulated in poly-lactide-glycolide beads (Southern Research  
Institute, Birmingham, AL) (Lmeif/PLG). Groups of mice were also immunized with  
10 LmeIF4A encapsulated in PLG beads (ova plus Lmeif/PLG) or PLG alone. The size of  
beads used for the experiment was in the range of 1 to 10 µm.

Three weeks after immunization, mice were sacrificed and the spleens  
removed. To generate effector cells, spleen cells were prepared and stimulated *in vitro*  
with the ova expressing EG7.ova cells, after irradiation (20,000 rad) for five days. The  
15 EG7.ova cell line was generated by transfecting the EL4 thymoma cell line (H-2<sup>b</sup>)  
(Infectious Disease Research Institute, Seattle, WA) with the ova cDNA-containing  
plasmid under the control of β-actin as described (Moore et al., *Cell* 54:777-785  
(1988)).

Priming was assessed by the presence of effectors capable of lysing <sup>51</sup>Cr  
20 labeled EG7.ova, without lysing <sup>51</sup>Cr labeled non-transfectant parental EL4 cells. The  
results are shown in Figure 13, and are expressed as % cytotoxicity as a function of  
effector to target cell ratio. These results demonstrate that mice immunized with  
soluble ovalbumin in combination with LmeIF4A show an excellent CTL response  
which killed target cells specifically. No specific CTL elicitation could be detected in  
25 mice immunized with either LmeIF4A, PLG or soluble ovalbumin alone.

### EXAMPLE 13

#### Stimulation of Trinitrophenol-specific Antibodies

30 This example illustrates the enhancement of anti-trinitrophenol (TNP)  
antibodies using LmeIF4A.

C57BL/6 mice were immunized (day 0) intraperitoneally with 3 µg  
TNP-KLH combined with either alum, LmeIF4A (75 µg) or with saline. The mice were  
bled on day 10, boosted (same as primary) on day 21 and bled on day 26. The sera were  
35 tested for TNP specific, isotype specific antibody responses by enzyme-linked  
immunosorbant assay (ELISA). Data are expressed, in Figure 14, as the log of the

dilution of each antiserum that gave a 50 percent of maximum OD in each ELISA, as calculated by linear regression analysis.

These results show a significant enhancement of antibody response by LmeIF4A, as compared to immunization with antigen alone. Of particular interest, no  
5 noticeable increase in IgE, the antibody class most associated with the Th2 response, was found. In addition, primary IgM and IgG antibody responses were enhanced after a single injection of TNP-KLH (3  $\mu$ g) with LmeIF4A (75  $\mu$ g), as described above.

#### EXAMPLE 14

##### 10 Enhancement of Production of MUC-1 Specific Antibodies

This example illustrates the use of LmeIF4A to enhance the production of MUC-1 specific antibodies.

C57BL/6 mice were immunized with human tumor antigen MUC-1 in  
15 various combinations with LmeIF4A. Groups included mice immunized with soluble MUC-1 with or without soluble LmeIF4A and MUC-1 immobilized on PLG beads with or without soluble LmeIF4A. For MUC-1/PLG experiments, two separate bead sizes were used. One batch of beads were used for 1-10  $\mu$ m in size while the other ranged from 40-100  $\mu$ m. Ten, twenty and thirty days after immunization, mice were bled and  
20 the sera were tested for the presence of MUC-1 specific antibodies (IgG and IgM). The sera were also tested for the presence of  $\mu$ ,  $\gamma$ 2a,  $\gamma$ 2b and  $\gamma$ 1 isotypes.

The results, shown in Figure 15, demonstrate that, while one immunization with the MUC-1 or MUC-1 plus LmeIF4A was not sufficient to generate an anti-MUC-1 antibody response, LmeIF4A significantly enhanced the antibody  
25 response when MUC-1 was encapsulated in PLG beads. MUC-1 in small PLG beads induced strong anti-MUC-1 responses deductible as early as 10 days after the first immunization and the addition of LmeIF4A to the preparation further enhanced the antibody response. One immunization with MUC-1 in large PLG beads did not produce any detectable anti-MUC-1 antibodies within 10 days of immunization, but an amplified  
30 response was observed in mice immunized with MUC-1 in large PLG beads in combination with LmeIF4A. The isotope distribution of anti-MUC-1 antibodies consisted of IgM, IgG $\gamma$ 2a, IgG $\gamma$ 2b, and IgG $\gamma$ 1 with no IgE response detected.

EXAMPLE 15Enhancement of CTL Activity in Cultured Cells

This example illustrates enhancement of specific CTL activity by  
5 LmeIF4A in cultured cells.

Mice were immunized with 30 µg of soluble ovalbumin or the same concentration of ovalbumin encapsulated in PLG beads. After two weeks, spleen cells were removed from immunized mice and stimulated *in vitro* with no antigen, LmeIF4A alone (10 µg/ml) irradiated EG7.ova or irradiated EG7.ova plus LmeIF4A at the same  
10 concentration. The results are shown in Figure 16.

These results indicate that ovalbumin/PLG immunized mice can prime ovalbumin-specific CTL *in vivo* which can be detected by stimulating responder spleen cells with EG7.ova stimulator cells. Furthermore, CTL generation from ovalbumin/PLG primed spleen cells can be substantially augmented by the addition of  
15 LmeIF4A (10 µg/ml) to the EG7.ova containing cultures. It should be noted that in the first *in vitro* culture with EG7.ova or EG7.ova plus LmeIF4A, total mononuclear cell counts did not differ (see Tables 2 and 3 below).

TABLE II  
LmeIF4A Enhances CTL Activities *Ex Vivo*

<u>Immunized With</u>	<u>Stimulated <i>ex vivo</i> with</u>	<u>Lytic Units (LU)/10<sup>6</sup> Mononuclear Cells</u>
None	No antigen	0
	LmeIF4A alone	0
	EG7.Ova	0
	EG7.Ova plus LmeIF4A	0
Bead alone	No antigen	0
	LmeIF4A alone	0
	EG7.Ova	0
	EG7.Ova plus LmeIF4A	0
Ova/bead	No antigen	0
	LmeIF4A alone	0
	EG7.Ova	2.5
	EG7.Ova plus LmeIF4A	16.6

5

TABLE III  
Recovery of Mononuclear Cells from LmeIF4A Containing Cultures

<u>Immunized With</u>	<u><i>In Vitro</i> Stimulated With</u>	<u>No. of Cells (LU)/10<sup>6</sup> Recovered/10<sup>7</sup> Mononuclear Cells Added</u>
Ova/PLGA	No Antigen	1.8
	LmeIF4A alone	2.4
	EG7.Ova	2.52
	EG7.Ova plus LmeIF4A	2.88
	Soluble Ova	3.2
	Soluble Ova plus LmeIF4A	2.6

10 We also evaluated whether LmeIF4A can be used to potentiate and expand antigen specific CTL activities *in vitro*. CTLs generated from ovalbumin/PLG-immunized mice discussed above were restimulated *in vitro* with either irradiated EG7.ova plus IL-2 or with EG7.ova plus IL-2 and LmeIF4A. After five days of culture, effector cells were tested against <sup>51</sup>Cr-labelled EL4 cells or EG7.ova cells.

15 The results, shown in Figure 17, indicate that ovalbumin-specific CTL restimulated with EG7.ova plus LmeIF4A and IL-2 killed EG7.ova better than the same effectors generated with EG7.ova plus IL-2 alone, but the difference does not appear to be significant. However, when the cell counts were taken, the culture with LmeIF4A

produced 2.5-fold higher cells than the cultures without LmeIF4A. These results, shown below in Table 4, are expressed in lytic units per culture, and demonstrate overall strength of LmeIF4A to expand specific CTL numbers *in vitro*.

5

TABLE IVRecovery of Mononuclear Cells from LmeIF4A Containing Cultures

<u>Immunized With</u>	<u><i>In Vitro</i> Stimulated With</u>	No. of Cells (10 <sup>6</sup> ) Recovered/10 <sup>6</sup> Mononuclear Cells Added	Total Lytic Units (LU50)/Culture
Ova/PLGA sm Nr	EG7.Ova plus IL-2	6.4	75.2
	EG7.Ova plus IL-2 and LmeIF4A	16	388.8

EXAMPLE 16

10

Augmentation of the Induction of Alloreactive CTL by LmeIF4A

This examples illustrates the induction of murine alloreactive CTL by LmeIF4A.

2.5 x 10<sup>6</sup> BALB/c spleen cells were cultured with 5 x 10<sup>6</sup> irradiated (3500 R) C57BL/6 spleen cells in 2 ml of RPMI:FCS in the presence or absence of 10 µg/ml gel-purified LmeIF4A. Cultures were harvested on day 5, washed and tested at different effector cell concentrations (culture fraction) for cytolytic activity against <sup>51</sup>Cr labelled EL4 cells (2,000/well) in a 4 hr release assay in 200 µl cultures. Data are expressed, in Figure 18, as percent specific release, which is calculated as:

20

$$\frac{100 \times (\text{CPM experimental}) - (\text{CPM spontaneous})}{(\text{CPM maximum}) - (\text{CPM spontaneous})}$$

These results indicate that LmeIF4A is capable of significantly enhancing alloreactive CTL. Taken together with the other results described above, the *Leishmania* eIF4A homologue appears to have potent adjuvant activity in a variety of assay systems. Furthermore, its ability to induce IL-12, together with its lack of toxicity, makes it a unique adjuvant.

25

EXAMPLE 17Tumor Regression After Administration of LmeIF4A and Antigen

Female C57BL/6 (H-2<sup>b</sup>) mice of 6-8 weeks of age were immunized once  
5 with a subcutaneous injection with 50 µg LmeIF4A either alone or 30 µg encapsulated  
in poly-lactide-glycolide beads (LbeIF/PLG), VSV peptides alone or in PLG beads,  
GM-CSF in PLG beads, or vehicle (PBS). N1 cells were generated from the EL4 cell  
by transfection of the VSV (vesicular stomatitis virus) nucleocapsid protein gene.  
Because the plasmid directing expression of the VSV nucleocapsid protein contains the  
10 neomycin resistance gene as a selectable marker, the cell line was maintained in a  
selective medium containing G418. Recombinant LmeIF4A was produced from *E. coli*  
transfectants and purified by HPLC fractionation. N1-specific peptide antigen was  
chemically synthesized and also purified by HPLC. Proteins were encapsulated into  
PLG microspheres by Southern Research Institute (Birmingham, AL).

15 Approximately  $2 \times 10^5$  N1 cells were inoculated intradermally into the  
right flank of C57BL/6 mice. After palpable tumors were established, mice were  
randomized into groups of five and given subcutaneous injections of the various  
peptides listed above on the opposite flank. Tumor growth was monitored by  
measuring the diameters of tumors every 2 or 3 days and converting the measurement  
20 into volume according to the formula  $V = 4/3 \pi r^3$ .

The efficacy of LmeIF4A in eliciting immune responses against tumor  
specific antigens was tested. In the murine tumor N1, the octameric antigenic peptide  
epitope RGYVYQGL is constitutively presented by the H-2K<sup>b</sup> molecule. As shown in  
Fig. 19, the injected combination of VSV/PLG and LmeIF4A/PLG resulted in  
25 significant suppression of tumor growth. Regression was not observed in the control  
mice or in mice receiving other combinations of antigens. A second study (Fig. 20)  
confirmed this result and also demonstrated that LmeIF4A is a much better adjuvant  
than GM-CSF. In this experiment, tumors started to regress one week after antigen  
injection in mice administered with the tumor specific VSV/PLG and LmeIF4A/PLG.  
30 Indeed, three out of five mice completely rejected their tumors in this particular study.  
In addition, soluble LeIF also had potent anti-tumor activity in combination with PLG  
encapsulated VSV peptide (Fig. 21).

In addition, the immune response generated through LmeIF4A and tumor  
antigen co-immunization was highly specific. The residual tumor mass in mice who did  
35 not completely reject N1 tumor was surgically removed. When these tumor cells were  
cultured in medium containing 0.2 mg/ml G418, they were killed two days later. In

contrast, original N1 cells used in the tumor inoculum were resistant to G418. Thus, the residual tumor was derived from N1 mutants that had lost the expression plasmid containing both the antigenic VSV sequence and the neomycin resistance genes. Therefore, LeIF is capable of boosting specific immune response against a predefined antigen, which, in turn, can lead to a therapeutic effect against tumors.

### EXAMPLE 18

#### Treatment of Established Tumors with LmeIF

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Female C57BL/6 mice were injected s.c. with  $2 \times 10^5$  Lewis lung carcinoma cells. By eight days following injection, tumors were detected in all mice. Mice were then divided into two groups of five mice each. On days 8, 10, 13, and 16 after tumor inoculation, mice were injected s.c., at a site distant from the tumor, with 0.2 ml saline or 50  $\mu$ g LmeIF in 0.2 ml saline. Tumor growth was measured on days 8, 10, 13, and 16. As shown in the following table, mice receiving LmeIF had substantially reduced tumor growth by day 16.

Table V

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#### Inhibition of Tumor Growth by LmeIF

<u>Group</u>	<u>Average tumor volume in mm<sup>3</sup></u>			
	<u>day 8</u>	<u>day 10</u>	<u>day 13</u>	<u>day 16</u>
Saline injected	5.21	12.61	41.06	283.53
Lmeif injected	6.79	10.83	26.75	69.48

25

### EXAMPLE 19

#### Ability of LeIF to Influence Cytokine Response and Generate LeIF Specific T Cell Clones

This Example illustrates the ability of LeIF to influence the Th1/Th2 cytokine responses and the generation of LeIF specific T cell clones in the absence of adjuvant.

A. Nature of LmeIF4A Specific T cell Responses in *Leishmania*-infected and uninfected BALB/c mice.

*Preparation of LmeIF4A.* cDNA encoding LmeIF4A was prepared as described above. Figure 22 shows a comparison of the predicted protein sequence of LmeIF4A (SEQ ID NO:4) with the homologous sequences from *L. braziliensis* (LbeIF4A; SEQ ID NO:2) mouse (MeIF; SEQ ID NO:5) and human (HeIF; SEQ ID NO:6) showing that LmeIF4A has the highest sequence homology to *L. braziliensis* eIF protein with 99.8% total homology (98.3% identity, 1.5% conservative substitution). Both LmeIF4A and LbeIF4A are of identical length with only seven amino acid residue substitutions (six being conservatively over their entire lengths (Figure 22). In contrast, LmeIF4A shows ~50% identity with the eIF proteins of mouse and human with the N terminal half representing the most variable portion between the eIF proteins of *Leishmania* and those of mouse and human. In fact, it was necessary to introduce gaps in the sequences to allow for maximum alignment between the *Leishmania* proteins and the mammalian homologues. Despite these differences, all four proteins have a series of conserved motifs arranged in identical order characteristic of the "DEAD box" family of RNA helicases. Two of these conserved sequences represent specialized versions of the A and B motifs previously described in other ATP binding proteins. The four amino sequence Asp-Glu-Ala-Asp (DEAD; SEQ ID NO:13) is part of the specialized version of the B motif. Motif I (Gly-Thr-Gly-Lys-Thr; SEQ ID NO:14) corresponds to the A-site of the nucleoside triphosphate (NTP)-binding motif and is found in most nucleotide-binding proteins including ATPases, kinases, and DNA and RNA helicases. Motif II corresponds to the B site of the NTP-binding motif which interacts through the invariant "D" residue with the Mg<sup>2+</sup> moiety of Mg-ATP.

Full length and overlapping *L. major* rLeIF proteins were expressed in *E. coli* with six histidine residues at the amino-terminal portion immediately following the initiator Met residue (N-terminal His-tag) of the pET plasmid vector (pET-17b) and a T7 RNA polymerase expression system (Novagen, Madison, WI). The cDNA was amplified by PCR from the initial cloned construct in pBSK(-) vector using specific oligonucleotides comprising 5' and 3' sequences. The specific oligonucleotide primers used for PCR amplification of the LeIF cDNA were as follows: 1) Full length sequence (amino acid residues 1 to 403: 5' [oligo 1 (SEQ ID NO:7) - CAA TTA CAT ATG CAT CAC CAT CAC CAT CAC ATG GCG CAG AAT GAT AAG ATC GCC] and 3' [oligo 403 (SEQ ID NO:8) - CAT GGA ATT CCG CTT ACT CGC CAA GGTAGG CAG C]); 2) amino acid residues 1 to 226: 5' oligo-1 as above and 3' [oligo 226 (SEQ ID NO:9) - CAT GGA ATT CTTA GTC GCG CAT GAA CTT CTT CGT CAG]; 3) amino acid



residues 196 to 403: 5' [oligo 196 (SEQ ID NO:10) - CAA TTA CAT ATG CAT CAC CAT CAC CAT CAC *TTC CGC TTC CTG CCG AAG GAC* ATC and 3' [oligo-403] as above; and 4) amino acid residues 129 to 261: 5' [oligo 129 (SEQ ID NO:11) - CAA TTA CAT ATG CAT CAC CAT CAC CAT CAC GAG ACC TTT GTC GGC GGC  
5 ACG CGC] and 3' [oligo 261 (SEQ ID NO:12) - CAT GGA ATT CTT ACA GGT CCA TCA GCG TGT CCA GCT T]. The 5' and 3' oligonucleotides contain Nde I and EcoR I restriction endonuclease sites (underlined) and primer sequences derived from LeIF sequence are indicated by italics with the ATG initiator and TAA terminator codons in bold. The PCR products were digested with Nde I and EcoR I and ligated into the  
10 poly-linker of pET-17b vector pre-digested with Nde I and EcoR I. *E. coli* strain BL21 (DE3) pLysE (Novagen) was used for high level expression.

The recombinant (His-Tag) antigens were purified from the insoluble inclusion body of 500 ml of IPTG induced batch cultures by affinity chromatography using the one step QIAexpress Ni-NTA Agarose matrix (QIAGEN, Chatsworth, CA) in  
15 the presence of 8M urea. Briefly, 20 ml of an overnight saturated culture of BL21 containing the pET construct was added into 500 ml of 2xYT media containing 50 µg/ml ampicillin and 34 µg/ml chloramphenicol, grown at 37°C with shaking. The bacterial cultures were induced with 2mM IPTG at an OD 560 of 0.3 and grown for an additional 3 hours (OD = 1.3 to 1.9). Cells were harvested from 500 ml batch cultures  
20 by centrifugation and resuspended in 20 ml of binding buffer (0.1 M sodium phosphate, pH 8.0; 10 mM Tris-HCl, pH 8.0) containing 2mM PMSF and 20 µg/ml leupeptin. *E. coli* was lysed by adding 15 mg of lysozyme and rocking for 30 minutes at 4°C following sonication (4 x 30 seconds), then spun at 12 k rpm for 30 minutes to pellet the inclusion bodies.

25 The inclusion bodies were washed three times in 1% CHAPS in 10 mM Tris-HCl (pH 8.0). This step greatly reduced the level of contaminating LPS. The inclusion body was finally solubilized in 20 ml of binding buffer containing 8 M urea or 8M urea was added directly into the soluble supernatant. Recombinant antigens with His-Tag residues were batch bound to Ni-NTA agarose resin (5 ml resin per 500 ml  
30 inductions) by rocking at room temperature for 1 hour and the complex passed over a column. The flow through was passed twice over the same column and the column washed three times with 30 ml each of wash buffer (0.1 M sodium phosphate and 10 mM Tris-HCL, pH 6.3) also containing 8 M urea. Bound protein was eluted with 30 ml of 100 mM imidazole in wash buffer and 5 ml fractions collected. Fractions  
35 containing the recombinant antigen were pooled, dialyzed against 10 mM Tris-HCl (pH 8.0) bound one more time to the Ni-NTA matrix, eluted and dialyzed in 10 mM

Tris-HCL (pH 7.8). The yield of recombinant protein varies from 25 - 150 mg per liter of induced bacterial culture with greater than 98% purity. Endotoxin levels were typically <10 EU/mg protein (*i.e.*, <1 ng LPS/mg).

The sizes of the expressed proteins (Figure 23) correlated well with their predicted molecular weights. The yield of purified rLmeIF4A was in the 50 to 100 mg/l range. The N-terminal sequence of all preparations were confirmed by direct sequencing the purified protein with a Procise 494 sequencer (Perkin Elmer/Applied Biosystems Division). Western blot analyses of rLmeIF4A with a rabbit anti-sera made against the *L. braziliensis* eIF4A protein revealed strong specific reactivity. Hereafter, LmeIF will be referred to generically as LeIF for *Leishmania* eIF protein.

*Immune responses to LmeIF by L. major infected BALB/c mice.* Infection of BALB/c mice with *L. major* is commonly used as a model system for cell-mediated immune regulation. These mice are widely accepted as developing a predominant Th2 profile by 7 to 10 days following infection thereby resulting in disease progression. Female, BALB/cByJ and BALB/cByJmn-*scid*/J mice were from Jackson Laboratory (Bar Harbour, ME) and were age-matched (4-6 weeks) within each of the following experiments.

BALB/c female mice (three per group) were immunized with recombinant 8E (a *L. braziliensis* antigen unrelated to LbeIF4A) or LmeIF in PBS. Each mouse received 70 µg of the indicated antigen in a final volume of 200 µl administered subcutaneously and distributed over three sites on the shaved flank. Inguinal, brachial, axillary, and periaortic lymph nodes were removed on day 10.

For proliferation, the lymph node cells were cultured for 72 hours at a density of  $4 \times 10^5$ /well in 96-well flat bottom plates in the presence of various concentrations of the indicated antigen. The cultures were pulsed with  $^3\text{H}$ -thymidine for the final 16 hours of culture and were harvested onto filters. The incorporation of radioactivity was determined using a Matrix 96 Direct Beta Counter (Packard Instrument Co., Inc).

For infection studies, *L. major* (Friedlan strain) promastigotes were cultured at 26°C in M199 (GibcoBRL, Gaithersburg, MD) containing 10% FCS (Hyclone, Logan, UT). For *in vitro* responses, BALB/c mice were infected with  $2 \times 10^5$  stationary phase *L. major* promastigotes in each hind footpad. At 10 and 28 days post-infection, animals were sacrificed and popliteal lymph nodes removed. Single cell suspensions were prepared from the nodes, plated at density of either  $2 \times 10^5$  cells per well (96 well flat bottom plates) for analysis of proliferative responses or at  $2 \times 10^6$  cells per well (24 well plate) for cytokine analyses. Cells were pulsed with 1 µCi of

[<sup>3</sup>H]-thymidine after 72 hours of culture and incorporation of radioactivity was determined approximately 16 hours later. Levels of cytokines (IFN- $\gamma$  and IL-4 ) secreted into the culture supernatants after 72 hours of culture were measured by ELISA.

5           The levels of IFN- $\gamma$ , IL-10, and IL-4 were determined by sandwich ELISA, using antibody pairs and procedures available from PharMingen. All determinations of cytokine levels were derived by testing serial dilutions of the supernatants. Standard curves were generated using recombinant mouse cytokines available from Immunex (IL4) or Genzyme (IL-10 and IFN- $\gamma$ ). The ELISAs for IFN- $\gamma$  and IL-10 were sensitive to 100 pg/ml of the appropriate cytokine and the ELISA for IL4 was sensitive to 20 pg/ml. Hamster anti-CD3 (500A2, gift of Dr. J. P. Allison, U. C. Berkeley, Berkeley, CA) was purified at Immunex. Since IL-10 production always correlated with IL-4 production, but IL-4 production did not always correlate with IL-10 production, the results for the Th profiles are shown in terms of IL-4 vs IFN- $\gamma$  production only.

*Results.* Culture supernatants of antigen-stimulated lymph node cells were analyzed for the production IL-4 and IFN- $\gamma$ . When cells from infected mice were stimulated *in vitro* with SLA, high levels of IL-4 and very little IFN- $\gamma$  were detected. However, the same cultures when stimulated with rLmeIF produced high levels of IFN- $\gamma$  and no IL4. The same result was obtained using mice at both 10 (Figure 24A) and 28 (Figure 24B) days after infection, by which time a clear Th2 pattern established in terms of disease progression. rLmeIF also elicited strong proliferative responses from draining lymph node cells of these *L. major*-infected BALB/c mice at both early (10 days) and late days) stages infection. By comparison, rLmSTI1 (a recently described immunogenic *L. major* antigen; Webb et al., *J. Immunol.* 157:5034-5041,1996) yielded a mixed cytokine profile.

          To complement the cytokine data, anti-LeIF antibody titers in sera from BALB/c mice at 28 days post infection were analyzed for anti-LeIF antibodies (Figure 24C). rLeIF was diluted in coating buffer (15 mM Na<sub>2</sub>HCO<sub>3</sub>, 28 mM NaH<sub>2</sub>CO<sub>3</sub>, pH 9.6) and plated onto Corning Easy Wash ELISA plates (Corning Glass Works, Corning, NY) at 1  $\mu$ g/wells and incubated overnight at 4°C. Plates were then blocked at room temperature with PBS containing 1% BSA for 1 hour). BALB/c mice were infected with 2x10<sup>5</sup> stationary phase *L. major* promastigotes in each hind footpad and were used as source of infection sera at 8 weeks post-infection. 100  $\mu$ l of sera diluted in PBS containing 0.1% BSA and 0.1% Tween-20 were added and incubated at room temperature for 30 minutes. Following removal of unbound antibodies (five washes

with PBS containing 0.1% Tween-20), bound antibodies were detected with goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Southern Biotechnology Associates Inc., Birmingham, AL). Plates were developed using TMB and read at 500 nm. Only very low levels of anti-LeIF antibody were detected at a serum dilution of 1:1,000. In contrast, the same mice develop extremely high LmSTI1-specific titers (>1:200,000) and high antibody titers to SLA. This demonstrates that LeIF induces relatively weak B cell responses during *L. major* infection.

rLeIF down regulates the in vitro production of SLA induced IL-4 from draining lymph nodes of infected Balb/c mice. To evaluate the ability of rLeIF to down regulate the production of IL-4 by lymphocytes of *L. major* infected mice, mice were infected for 28 days with *L. major*, followed by culturing of lymph node cells with SLA. This resulted in the production of IL4, but not IFN- $\gamma$  by the lymph cells. The addition of various concentrations of rLeIF and a fixed amount of SLA to the lymph node cultures caused a nearly complete abrogation of the SLA-induced IL-4 secretion in a dose dependent manner (Figure 25). It was also observed that SLA had no effect on LeIF induced IFN- $\gamma$  production.

These results indicate that lymph node cells from *Leishmania* infected BALB/c mice that are stimulated *in vitro* with soluble leishmanial antigen (SLA), produce a cytokine profile biased towards IL-4. However, the same cells stimulated with LmeIF4A produced high amounts of IFN- $\gamma$  and no detectable IL-4. The addition of LmeIF4A to SLA resulted in decreased IL-4 production, demonstrating the ability of LmeIF4A to down regulate a Th2 response.

B. Th Responses to LmeIF4A in Naive BALB/c Mice Following Immunization with LbeIF4A in the Absence of Added Adjuvant.

Because IL-12 is a key cytokine that favors the development of Th1 responses, the ability of LeIF to induce a LeIF specific Th1 profile in naive BALB/c mice in the absence of added adjuvant was evaluated. In this set of experiments, the *L. braziliensis* eIF homologue (LbeIF) was used as the immunizing antigen. Animals were primed with either rLbeIF or r8E (another recombinant leishmanial antigen which stimulates patient PBMC to produce high levels of IL-10) in PBS. Both antigens contain the same 4 kD,  $\beta$ -gal N-terminal fusion sequence.

Draining lymph node cells from these animals were first cultured for ten days at  $6 \times 10^6$ /well in a 2 ml volume in the presence of 0.5  $\mu$ g/ml 8E or 10  $\mu$ g/ml LmeIF. The short term T cell lines were then cloned by limiting dilution in the

presence of 8E or LmIF (0.5 or 10 mg/ml, respectively), irradiated BALB/c splenocytes ( $2.5 \times 10^5$ /well) and IL-2 (10  $\mu$ g/ml). Three weeks later the resulting clones were resuspended in a total volume of 300  $\mu$ l and were transferred into wells containing immobilized anti-CD3 (0.5  $\mu$ g/well of a 48 well plate). Supernatants were collected 48 hours later.

T cell lines were assayed for IFN- $\gamma$  and IL-4. It was found that ~90% of the T-cell clones isolated from LeIF-primed mice were Th1, producing IFN- $\gamma$  and no IL-4, while the remaining 10 % were Th0 producing both IFN- $\gamma$  and IL-4 (Figure 26). In contrast, clones from mice primed with 8E had a mixed cytokine profile.

These results suggest that the ability of LeIF to drive a specific Th1 response is mediated by an adjuvant activity of LeIF itself.

#### C. Ability of LmIF4A to Influence the Early Cytokine Profile in Splenocytes of SCID Mice.

To examine the activity of LeIF on spleen cells in the absence of T cells, the above experiments were repeated using splenocytes from SCID mice of two different genetic backgrounds both *Leishmania* resistant (C3H) and *Leishmania* susceptible (BALB/c). Briefly, cells from spleens of 6 week old female SCID mice were cultured at  $2 \times 10^6$  per well and stimulated with 10  $\mu$ g/ml with rLeIF or SLA. At 12, 24, and 72 hours, supernatants from the induced cultures were harvested and the levels of IFN- $\gamma$  measured. The results demonstrate that LeIF can stimulate the production of high levels of IFN- $\gamma$  by SCID mouse spleen cells in both genetic background (Figure 27A). In contrast SLA did not stimulate the production of detectable IFN- $\gamma$ . The IFN- $\gamma$  production induced by LeIF in SCID splenocytes was IL-12 mediated; the addition of anti-IL-12 antibody abrogated the production of IFN- $\gamma$  in these cultures (Figure 27 B). These results demonstrate that in SCID mice, LeIF can stimulate IL-12 production which would subsequently stimulate NK cells to produce IFN- $\gamma$ .

Within a further experiment, cytokine mRNA expression was evaluated in SCID mice splenocytes stimulated with rLeIF. Splenocytes from C3H SCID mice were cultured at  $5 \times 10^6$  cells/well (0.6ml) in the absence (-) or presence (+) of 10  $\mu$ g/ml of rLeIF for 24 hours. Total RNA was isolated and RT-PCR assays performed using cytokine specific primers as indicated in Figure 28. The results indicate that LeIF increases the level of IFN- $\gamma$  and IL-18 mRNA, but not IL-10 mRNA.

#### D. Synergistic Effect between LeIF and Cytokines

A synergistic effect of LeIF and certain cytokines was identified in SCID mouse splenocytes. In one experiment, SCID mouse splenocytes ( $2 \times 10^6/\text{ml}$ ) were stimulated with IL-18 and LeIF ( $10 \mu\text{g}/\text{ml}$ ) for 72 hours. The supernatant was harvested and the level of IFN- $\gamma$  assayed as described above. The results, presented in Figure 29, show that the level of IFN- $\gamma$  increases with increasing amounts of IL-18 in the presence of LeIF.

In a similar experiment, SCID mouse splenocytes ( $2 \times 10^6/\text{ml}$ ) were stimulated with IL-18 and IL-15 (10 or 100 ng/ml) in the presence or absence of LeIF ( $10 \mu\text{g}/\text{ml}$ ) for 72 hours. The supernatant was harvested and the level of IFN- $\gamma$  assayed as described above. The results, presented in Figures 30 and 31, show that the presence of IL-15 further enhances the LeIF-induced IFN- $\gamma$  production in the presence of IL-18.

Within further experiments, IL-15 and IL-18 were shown to increase NK cell cytotoxic activity of SCID mouse splenocytes stimulated with LeIF. SCID mouse splenocytes were incubated with either IL-15 or IL-18 (100 ng/ml), IL-12 (10 U/ml) or both in the presence or absence of LeIF ( $10 \mu\text{g}/\text{ml}$ ) for 24 hours. NK-cell activity was assayed by  $^{51}\text{Cr}$  release from YAC-1 as a target cell. The results in Figures 32 and 33 shows that LeIF enhances the cytotoxic activity of the NK cells stimulated by IL-15 or IL-18.

#### 20 D. Initial Mapping of Active Regions

The mapping of the active region(s) was initiated by constructing three overlapping LeIF deletions. The recombinant clones were designed to encode the N-terminal half (amino acid residues 1-226), the middle portion (residues 129-261) and the C-terminal half (residues 196-403) of LeIF; (Fig. 2B). Purified proteins were subsequently evaluated for their ability to stimulate IFN- $\gamma$  production splenocytes from C3H SCID mice by harvesting the cultures at 72 hours post stimulation with LeIF. As shown in Figure 27C, the N-terminal half of LeIF retained the ability to induce IFN- $\gamma$  with levels that were generally higher than observed with full length LeIF. The middle fragment (129-261) stimulated the production of very low levels of IFN- $\gamma$  in the same assay. No detectable IFN- $\gamma$  was found in supernatants of splenocytes following stimulation with the C-terminal half of LeIF.

The results described herein indicate that LbeIF4A and LmeIF4A polypeptides are powerful and selective activators for Th1 cytokines that may be used as a prophylactic and therapeutic vaccine antigen for leishmaniasis. Such polypeptides act through T cell dependent and independent pathways to stimulate production of IL-

12. In the T cell dependent mechanism (as in the generation of LeIF specific Th1 clones), activated LeIF specific CD4<sup>+</sup> T cells would induce IL-12 production from macrophage through CD40-ligand CD40 interactions. However, the ability of LeIF to stimulate splenocytes from SCID mice to produce IFN- $\gamma$  is a novel finding and suggests that in this T cell independent pathway, LeIF acts by stimulating IL-12 production directly from monocyte/macrophages which would subsequently stimulate NK cells to produce IFN- $\gamma$ . This surprising finding demonstrates that LeIF can, in the absence of other leishmanial antigens, adjuvant its own T cell response and may help explain the reasons for a predominant bias towards the generation of LeIF specific Th1 clones.

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From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for the purpose of illustration, various modifications may be made without deviating from the spirit and scope of the invention.

Claims

We claim:

1. A composition comprising an antigen and an LbeIF4A polypeptide comprising an amino acid sequence encoded by a DNA sequence selected from the group consisting of:
  - (a) nucleotides 115 through 1323 of SEQ ID NO:1;
  - (b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 115 through 1323 of SEQ ID NO:1 under moderately stringent conditions, wherein the DNA sequence encodes a polypeptide that stimulates a Th1 immune response in peripheral blood mononuclear cells obtained from a *Leishmania*-infected individual, for use in the manufacture of a medicament for enhancing or eliciting an immune response to an antigen in a patient.
2. The composition of claim 1 wherein the LbeIF4A polypeptide comprises amino acids 49-403 of SEQ ID NO:2.
3. The composition of claim 1 wherein the LbeIF4A polypeptide comprises amino acids 1-403 of SEQ ID NO:2 or a portion thereof.
4. The composition of claim 1 wherein the LbeIF4A polypeptide additionally comprises an immunoglobulin Fc region.
5. A composition comprising an antigen and an LmeIF4A polypeptide comprising an amino acid sequence encoded by a DNA sequence selected from the group consisting of:
  - (a) nucleotides 117 through 1325 of SEQ ID NO:3; and
  - (b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 117 through 1325 of SEQ ID NO:3 under moderately stringent conditions, wherein the DNA sequence encodes a polypeptide that stimulates a Th1 immune response in peripheral blood mononuclear cells obtained from a *Leishmania*-infected individual, for use in the manufacture of medicament for enhancing or eliciting an immune response to an antigen in a patient.
6. The composition of claim 5 wherein the LmeIF4A polypeptide comprises amino acids 49-403 of SEQ ID NO:4.



7. The composition of claim 5 wherein the LmeIF4A polypeptide comprises amino acids 1-403 of SEQ ID NO:4 or a portion thereof.

8. The composition of claim 5 wherein the LmeIF4A polypeptide additionally comprises an immunoglobulin Fc region.

9. A composition comprising an antigen and an LbeIF4A polypeptide comprising amino acids 1-403 of SEQ ID NO:2 or a variant thereof that differs only in conservative substitutions and/or modifications, for use in the manufacture of a medicament for enhancing or eliciting an immune response to an antigen in a patient.

10. A composition comprising an antigen and an LmeIF4A polypeptide comprising amino acids 1-403 of SEQ ID NO:4 or a variant thereof that differs only in conservative substitutions and/or modifications, for use in the manufacture of a medicament for enhancing or eliciting an immune response to an antigen in a patient.

11. The composition of any of claims 1-10 wherein the antigen and the LbeIF4A or LmeIF4A polypeptide are present in the same composition.

12. The composition of any of claims 1-10 wherein the antigen is encapsulated in biodegradable microspheres.

13. The composition of any of claims 1-10 wherein the LbeIF4A or LmeIF4A polypeptide is encapsulated in or associated with the surface of biodegradable microspheres.

14. The composition of any of claims 1-10 wherein the antigen and the LbeIF4A or LmeIF4A polypeptide are encapsulated in biodegradable microspheres.

15. A composition comprising a DNA vaccine and an LbeIF4A polypeptide comprising an amino acid sequence encoded by a DNA sequence selected from the group consisting of:

- (a) nucleotides 115 through 1323 of SEQ ID NO:1;
- (b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 115 through 1323 of SEQ ID NO:1 under moderately stringent

conditions, wherein the DNA sequence encodes a polypeptide that stimulates a Th1 immune response in peripheral blood mononuclear cells obtained from a *Leishmania*-infected individual, for use in the manufacture of a medicament for enhancing or eliciting an immune response to a DNA vaccine in a patient.

16. The composition of claim 15 wherein the LbeIF4A polypeptide comprises amino acids 49-403 of SEQ ID NO:2.

17. The composition of claim 15 wherein the LbeIF4A polypeptide comprises amino acids 1-403 of SEQ ID NO:2 or a portion thereof.

18. The composition of claim 15 wherein the LbeIF4A polypeptide additionally comprises an immunoglobulin Fc region.

19. A composition comprising a DNA vaccine and an LmeIF4A polypeptide comprising an amino acid sequence encoded by a DNA sequence selected from the group consisting of:

- (a) nucleotides 117 through 1325 of SEQ ID NO:3; and
- (b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 117 through 1325 of SEQ ID NO:3 under moderately stringent conditions, wherein the DNA sequence encodes a polypeptide that stimulates a Th1 immune response in peripheral blood mononuclear cells obtained from a *Leishmania*-infected individual, for use in the manufacture of a medicament for enhancing or eliciting an immune response to a DNA vaccine in a patient.

20. The composition of claim 19 wherein the LmeIF4A polypeptide comprises amino acids 49-403 of SEQ ID NO:4.

21. The composition of claim 19 wherein the LmeIF4A polypeptide comprises amino acids 1-403 of SEQ ID NO:4 or a portion thereof.

22. The composition of claim 19 wherein the LmeIF4A polypeptide additionally comprises an immunoglobulin Fc region.

23. A composition comprising a DNA vaccine and an LbeIF4A polypeptide comprising amino acids 1-403 of SEQ ID NO:2 or a variant thereof that differs

only in conservative substitutions and/or modifications, for use in the manufacture of a medicament for enhancing or eliciting an immune response to a DNA vaccine in a patient.

24. A composition comprising a DNA vaccine and an LmeIF4A polypeptide comprising amino acids 1-403 of SEQ ID NO:4 or a variant thereof that differs only in conservative substitutions and/or modifications, for use in the manufacture of a medicament for enhancing or eliciting an immune response to a DNA vaccine in a patient.

25. The composition of any of claims 15-24 wherein the DNA vaccine and the LbeIF4A or LmeIF4A polypeptide are present in the same composition.

26. The composition of any of claims 15-24 wherein the DNA vaccine is encapsulated in biodegradable microspheres.

27. The composition of any of claims 15-24 wherein the LbeIF4A or LmeIF4A polypeptide is encapsulated in or associated with the surface of biodegradable microspheres.

28. The composition of any of claims 15-24 wherein the DNA vaccine and the LbeIF4A or LmeIF4A polypeptide are encapsulated in biodegradable microspheres.

29. An LbeIF4A polypeptide comprising an amino acid sequence encoded by a DNA sequence selected from the group consisting of:

- (a) nucleotides 115 through 1323 of SEQ ID NO:1;
- (b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 115 through 1323 of SEQ ID NO:1 under moderately stringent conditions, wherein the DNA sequence encodes a polypeptide that stimulates a Th1 immune response in peripheral blood mononuclear cells obtained from a *Leishmania*-infected individual, for use in the manufacture of a medicament for enhancing or eliciting an immune response to an antigen in a patient.

30. An LmeIF4A polypeptide comprising an amino acid sequence encoded by a DNA sequence selected from the group consisting of:

- (a) nucleotides 117 through 1325 of SEQ ID NO:3; and
- (b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 117 through 1325 of SEQ ID NO:3 under moderately stringent

conditions, wherein the DNA sequence encodes a polypeptide that stimulates a Th1 immune response in peripheral blood mononuclear cells obtained from a *Leishmania*-infected individual, for use in the manufacture of a medicament for enhancing or eliciting an immune response to an antigen in a patient.

31. A method of enhancing or eliciting an immune response in a biological sample, comprising contacting a biological sample with an antigen and an LbeIF4A polypeptide comprising an amino acid sequence encoded by a DNA sequence selected from the group consisting of:

- (a) nucleotides 115 through 1323 of SEQ ID NO:1;
- (b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 115 through 1323 of SEQ ID NO:1 under moderately stringent conditions, wherein the DNA sequence encodes a polypeptide that stimulates a Th1 immune response in peripheral blood mononuclear cells obtained from a *Leishmania*-infected individual; and

wherein the biological sample comprises cells selected from the group consisting of peripheral blood mononuclear cells, monocytes, B cells, dendritic cells and combinations thereof.

32. A method of enhancing or eliciting an immune response in a biological sample, comprising contacting a biological sample with an antigen and an LmeIF4A polypeptide comprising an amino acid sequence encoded by a DNA sequence selected from the group consisting of:

- (a) nucleotides 117 through 1325 of SEQ ID NO:3; and
- (b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 117 through 1325 of SEQ ID NO:3 under moderately stringent conditions, wherein the DNA sequence encodes a polypeptide that stimulates a Th1 immune response in peripheral blood mononuclear cells obtained from a *Leishmania*-infected individual; and

wherein the biological sample comprises cells selected from the group consisting of peripheral blood mononuclear cells, monocytes, B cells, dendritic cells and combinations thereof.

33. A method of enhancing or eliciting an immune response in a biological sample, comprising contacting a biological sample with a DNA vaccine and an LbeIF4A

polypeptide comprising an amino acid sequence encoded by a DNA sequence selected from the group consisting of:

- (a) nucleotides 115 through 1323 of SEQ ID NO:1;
- (b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 115 through 1323 of SEQ ID NO:1 under moderately stringent conditions, wherein the DNA sequence encodes a polypeptide that stimulates a Th1 immune response in peripheral blood mononuclear cells obtained from a *Leishmania*-infected individual; and

wherein the biological sample comprises cells selected from the group consisting of peripheral blood mononuclear cells, monocytes, B cells, dendritic cells and combinations thereof.

34. A method of enhancing or eliciting an immune response in a biological sample, comprising contacting a biological sample with a DNA vaccine and an LmeIF4A polypeptide comprising an amino acid sequence encoded by a DNA sequence selected from the group consisting of:

- (a) nucleotides 117 through 1325 of SEQ ID NO:3; and
- (b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 117 through 1325 of SEQ ID NO:3 under moderately stringent conditions, wherein the DNA sequence encodes a polypeptide that stimulates a Th1 immune response in peripheral blood mononuclear cells obtained from a *Leishmania*-infected individual; and

wherein the biological sample comprises cells selected from the group consisting of peripheral blood mononuclear cells, monocytes, B cells, dendritic cells and combinations thereof.

35. A method of enhancing or eliciting an immune response in a biological sample, comprising contacting a biological sample with an LbeIF4A polypeptide comprising an amino acid sequence encoded by a DNA sequence selected from the group consisting of:

- (a) nucleotides 115 through 1323 of SEQ ID NO:1;
- (b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 115 through 1323 of SEQ ID NO:1 under moderately stringent conditions, wherein the DNA sequence encodes a polypeptide that stimulates a Th1 immune response in peripheral blood mononuclear cells obtained from a *Leishmania*-infected individual; and

wherein the biological sample comprises cells selected from the group consisting of peripheral blood mononuclear cells, monocytes, B cells, dendritic cells and combinations thereof.

36. A method of enhancing or eliciting an immune response in a biological sample, comprising contacting a biological sample with an LmeIF4A polypeptide comprising an amino acid sequence encoded by a DNA sequence selected from the group consisting of:

- (a) nucleotides 117 through 1325 of SEQ ID NO:3; and
- (b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 117 through 1325 of SEQ ID NO:3 under moderately stringent conditions, wherein the DNA sequence encodes a polypeptide that stimulates a Th1 immune response in peripheral blood mononuclear cells obtained from a *Leishmania*-infected individual; and

wherein the biological sample comprises cells selected from the group consisting of peripheral blood mononuclear cells, monocytes, B cells, dendritic cells and combinations thereof.

37. A composition comprising a tumor antigen and an LbeIF4A polypeptide comprising an amino acid sequence encoded by a DNA sequence selected from the group consisting of:

- (a) nucleotides 115 through 1323 of SEQ ID NO:1;
- (b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 115 through 1323 of SEQ ID NO:1 under moderately stringent conditions, wherein the DNA sequence encodes a polypeptide that stimulates a CTL response in peripheral blood mononuclear cells obtained from a *Leishmania*-infected individual, for use in the manufacture of a medicament for enhancing or eliciting an immune response to a tumor in a patient.

38. The composition of claim 37 wherein the LbeIF4A polypeptide comprises amino acids 49-403 of SEQ ID NO:2.

39. A composition comprising a tumor antigen and an LbeIF4A polypeptide comprising an amino acid sequence encoded by a DNA sequence selected from the group consisting of:

- (a) nucleotides 117 through 1325 of SEQ ID NO:3; and

(b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 117 through 1325 of SEQ ID NO:3 under moderately stringent conditions, wherein the DNA sequence encodes a polypeptide that stimulates a CTL response in peripheral blood mononuclear cells obtained from a *Leishmania*-infected individual, for use in the manufacture of a medicament for enhancing or eliciting an immune response to a tumor in a patient.

40. The composition of claim 39 wherein the LmeIF4A polypeptide comprises amino acids 49-403 of SEQ ID NO:4.

41. A composition comprising a tumor antigen and an LbeIF4A polypeptide comprising amino acids 49-403 of SEQ ID NO:2, or a variant thereof that differs only in conservative substitutions and/or modifications, for use in the manufacture of a medicament for enhancing or eliciting an immune response to a tumor in a patient.

42. A composition comprising a tumor antigen and an LmeIF4A polypeptide comprising amino acids 49-403 of SEQ ID NO:4, or a variant thereof that differs only in conservative substitutions and/or modifications, for use in the manufacture of a medicament for enhancing or eliciting an immune response to a tumor in a patient.

43. A composition comprising a DNA vaccine encoding a tumor antigen and an LbeIF4A polypeptide comprising an amino acid sequence encoded by a DNA sequence selected from the group consisting of:

- (a) nucleotides 115 through 1323 of SEQ ID NO:1;
- (b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 115 through 1323 of SEQ ID NO:1 under moderately stringent conditions, wherein the DNA sequence encodes a polypeptide that stimulates a CTL response in peripheral blood mononuclear cells obtained from a *Leishmania*-infected individual, for use in the manufacture of a medicament for enhancing or eliciting an immune response to a tumor in a patient.

44. The composition of claim 43 wherein the LbeIF4A polypeptide comprises amino acids 49-403 of SEQ ID NO:2.

45. A composition comprising a DNA vaccine encoding a tumor antigen and an LbeIF4A polypeptide comprising an amino acid sequence encoded by a DNA sequence selected from the group consisting of:

- (a) nucleotides 117 through 1325 of SEQ ID NO:3; and
- (b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 117 through 1325 of SEQ ID NO:3 under moderately stringent conditions, wherein the DNA sequence encodes a polypeptide that stimulates a CTL response in peripheral blood mononuclear cells obtained from a *Leishmania*-infected individual, for use in the manufacture of a medicament for enhancing or eliciting an immune response to a tumor in a patient.

46. The composition of claim 45 wherein the LmeIF4A polypeptide comprises amino acids 49-403 of SEQ ID NO:4.

47. A composition comprising a DNA vaccine encoding a tumor antigen and an LbeIF4A polypeptide comprising amino acids 49-403 of SEQ ID NO:2, or a variant thereof that differs only in conservative substitutions and/or modifications, for use in the manufacture of a medicament for enhancing or eliciting an immune response to a tumor in a patient.

48. A composition comprising a DNA vaccine encoding a tumor antigen and an LmeIF4A polypeptide comprising amino acids 49-403 of SEQ ID NO:4, or a variant thereof that differs only in conservative substitutions and/or modifications, for use in the manufacture of a medicament for enhancing or eliciting an immune response to a tumor in a patient.

49. An LbeIF4A polypeptide, comprising:

- (a) nucleotides 115 through 1323 of SEQ ID NO:1;
- (b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 115 through 1323 of SEQ ID NO:1 under moderately stringent conditions, wherein the DNA sequence encodes a polypeptide that stimulates a CTL response in human peripheral blood mononuclear cells, for use in the manufacture of a medicament for treating a tumor in a patient.

50. An LmeIF4A polypeptide, comprising:

- (a) nucleotides 117 through 1325 of SEQ ID NO:3; and



(b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 117 through 1325 of SEQ ID NO:3 under moderately stringent conditions, wherein the DNA sequence encodes a polypeptide that stimulates a CTL response in human peripheral blood mononuclear cells, for use in the manufacture of a medicament for treating a tumor in a patient.

51. The composition of any of claims 39-40 wherein the LbeIF4A or LmeIF4A polypeptide is encapsulated in or associated with the surface of a biodegradable microsphere.

52. The composition of either of claims 49 or 50, further comprising a therapeutic reagent selected from the group consisting of cytokines and chemotherapeutic agents.

53. A composition comprising an antigen and a viral vector directing the expression of an LbeIF4A or LmeIF4A polypeptide in patient cells infected with the viral vector, for use in the manufacture of a medicament for enhancing or eliciting an immune response to an antigen in a patient.

54. A composition comprising an antigen and a nucleic acid molecule directing the expression of an LbeIF4A or LmeIF4A polypeptide in patient cells transfected with the nucleic acid molecule, for use in the manufacture of a medicament for enhancing or eliciting an immune response to an antigen in a patient.

55. A composition comprising a DNA vaccine and a viral vector directing the expression of an LbeIF4A or LmeIF4A polypeptide in patient cells infected with the viral vector, for use in the manufacture of a medicament for enhancing or eliciting an immune response to a DNA vaccine in a patient.

56. A composition comprising a DNA vaccine and a nucleic acid molecule directing the expression of an LbeIF4A or LmeIF4A polypeptide in patient cells transfected with the nucleic acid molecule, for use in the manufacture of a medicament for enhancing or eliciting an immune response to a DNA vaccine in a patient.

57. A composition comprising a viral vector directing the expression of an LbeIF4A or LmeIF4A polypeptide in patient cells infected with the viral vector, for use in the

manufacture of a medicament for enhancing or eliciting an immune response to an antigen in a patient.

58. A composition comprising a nucleic acid molecule directing the expression of an LbeIF4A or LmeIF4A polypeptide in patient cells transfected with the nucleic acid molecule, for use in the manufacture of a medicament for enhancing or eliciting an immune response to an antigen in a patient.

59. A composition comprising a tumor antigen and a viral vector directing the expression of an LbeIF4A or LmeIF4A polypeptide in patient cells infected with the viral vector, for use in the manufacture of a medicament for enhancing or eliciting an immune response to a tumor in a patient.

60. A composition comprising a tumor antigen and a nucleic acid molecule directing the expression of an LbeIF4A or LmeIF4A polypeptide in patient cells transfected with the nucleic acid molecule, for use in the manufacture of a medicament for enhancing or eliciting an immune response to a tumor in a patient.

61. A composition comprising a DNA vaccine encoding a tumor antigen and a viral vector directing the expression of an LbeIF4A or LmeIF4A polypeptide in patient cells infected with the viral vector, for use in the manufacture of a medicament for enhancing or eliciting an immune response to a tumor in a patient.

62. A composition comprising a DNA vaccine encoding a tumor antigen and a nucleic acid molecule directing the expression of an LbeIF4A or LmeIF4A polypeptide in patient cells transfected with the nucleic acid molecule, for use in the manufacture of a medicament for enhancing or eliciting an immune response to a tumor in a patient.

63. A composition comprising a viral vector directing the expression of an LbeIF4A or LmeIF4A polypeptide in patient cells infected with the viral vector, for use in the manufacture of a medicament for treating a tumor in a patient.

64. A composition comprising a nucleic acid molecule directing the expression of an LbeIF4A or LmeIF4A polypeptide in patient cells transfected with the nucleic acid molecule, for use in the manufacture of a medicament for treating a tumor in a patient.

65. The composition of any of claims 53-64 wherein the polypeptide is an LbeIF4A polypeptide comprising an amino acid sequence encoded by a DNA sequence selected from the group consisting of:

- (a) nucleotides 115 through 1323 of SEQ ID NO:1;
- (b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 115 through 1323 of SEQ ID NO:1 under moderately stringent conditions, wherein the DNA sequence encodes a polypeptide that stimulates a Th1 immune response in peripheral blood mononuclear cells obtained from a *Leishmania*-infected individual.

66. The composition of claim 65 wherein the LbeIF4A polypeptide comprises amino acids 49-403 of SEQ ID NO:2.

67. The composition of claim 65 wherein the LbeIF4A polypeptide comprises amino acids 1-403 of SEQ ID NO:2 or a portion thereof.

68. The composition of any of claims 53-64 wherein the polypeptide is an LmeIF4A polypeptide comprising an amino acid sequence encoded by a DNA sequence selected from the group consisting of:

- (a) nucleotides 117 through 1325 of SEQ ID NO:3; and
- (b) DNA sequences that hybridize to a nucleotide sequence complementary to nucleotides 117 through 1325 of SEQ ID NO:3 under moderately stringent conditions, wherein the DNA sequence encodes a polypeptide that stimulates a Th1 immune response in peripheral blood mononuclear cells obtained from a *Leishmania*-infected individual.

69. The composition of claim 68 wherein the LmeIF4A polypeptide comprises amino acids 49-403 of SEQ ID NO:4.

70. The composition of claim 68 wherein the LmeIF4A polypeptide comprises amino acids 1-403 of SEQ ID NO:4 or a portion thereof.

71. The composition of claim 53 or 59 wherein the viral vector also directs the expression of the antigen.

72. The composition of claim 54 or 60 wherein the nucleic acid molecule also directs the expression of the antigen.

73. An (a) LeIF4A polypeptide; (b) a nucleic acid molecule directing the expression of an LeIF4A polypeptide in patient cells transfected with the nucleic acid molecule or (c) a viral vector directing the expression of an LeIF4A polypeptide in patient cells infected with the viral vector, for use in the manufacture of a medicament for treating a Th2-mediated disease in a patient.

74. A composition according to claim 73, wherein the Th2-mediated disease is selected from the group consisting of asthma, allergy or Helminth infection.

75. An (a) LeIF4A polypeptide; (b) a nucleic acid molecule directing the expression of an LeIF4A polypeptide in patient cells transfected with the nucleic acid molecule or (c) a viral vector directing the expression of an LeIF4A polypeptide in patient cells infected with the viral vector, for use in the manufacture of a medicament for decreasing production of one or more Th2-associated cytokines in a patient.

76. A composition according to claim 75, wherein the Th2-associated cytokine is IL-4.

77. A composition according to claim 75, wherein the Th2-associated cytokine is IL-5.

78. An (a) LeIF4A polypeptide; (b) a nucleic acid molecule directing the expression of an LeIF4A polypeptide in patient cells transfected with the nucleic acid molecule or (c) a viral vector directing the expression of an LeIF4A polypeptide in patient cells infected with the viral vector, for use in the manufacture of a medicament for stimulating or enhancing IL-18 production in a patient.

79. A method for enhancing or eliciting an immune response to an antigen in a biological sample, comprising contacting a biological sample with an LeIF4A polypeptide in combination with one or more Th1-associated cytokines.

80. A composition according to claim 79, wherein the Th1-associated cytokines are selected from the group consisting of IL-2, IL-12, IL-15 and IL-18.

81. A composition comprising one or more Th1-associated cytokines in combination with (a) an LeIF4A polypeptide; (b) a nucleic acid molecule directing the expression of an LeIF4A polypeptide in patient cells transfected with the nucleic acid molecule or (c) a viral vector directing the expression of an LeIF4A polypeptide in patient cells infected with the viral vector, for use in the manufacture of a medicament for enhancing or eliciting an immune response to an antigen in a patient.

82. A composition according to claim 81, wherein the Th1-associated cytokines are selected from the group consisting of IL-2, IL-12, IL-15 and IL-18.

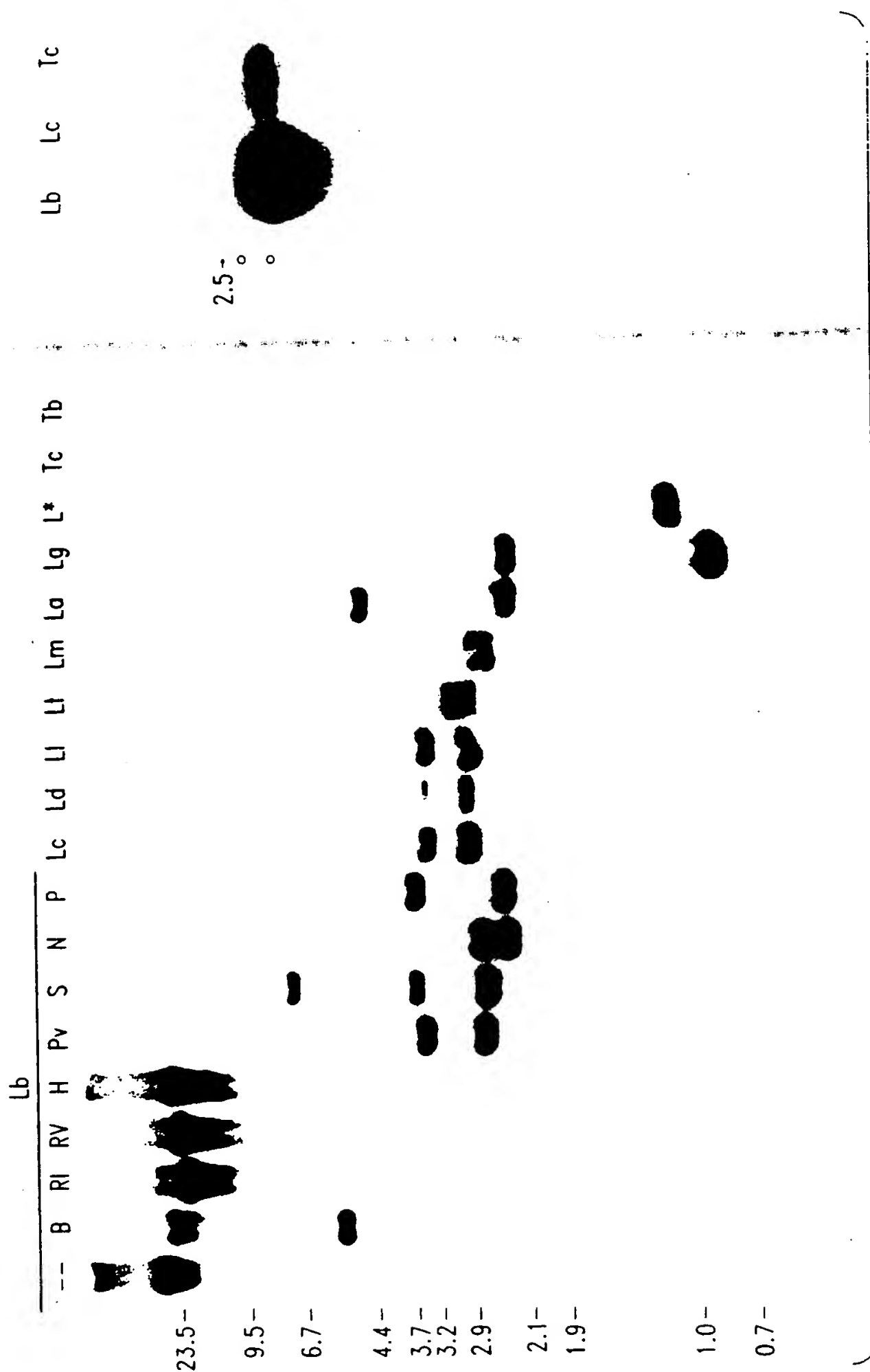
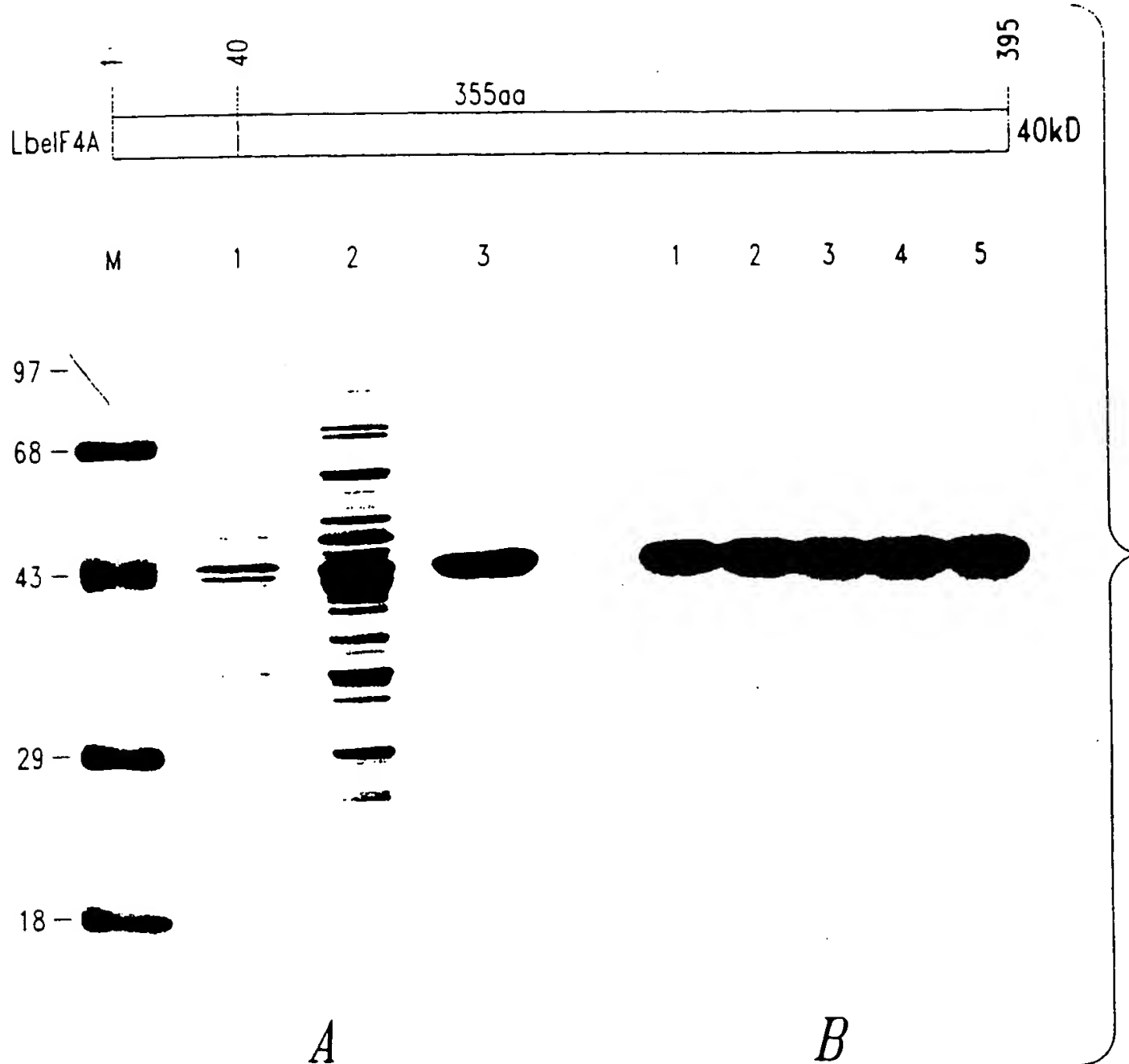


Fig. 1



*Fig. 2*

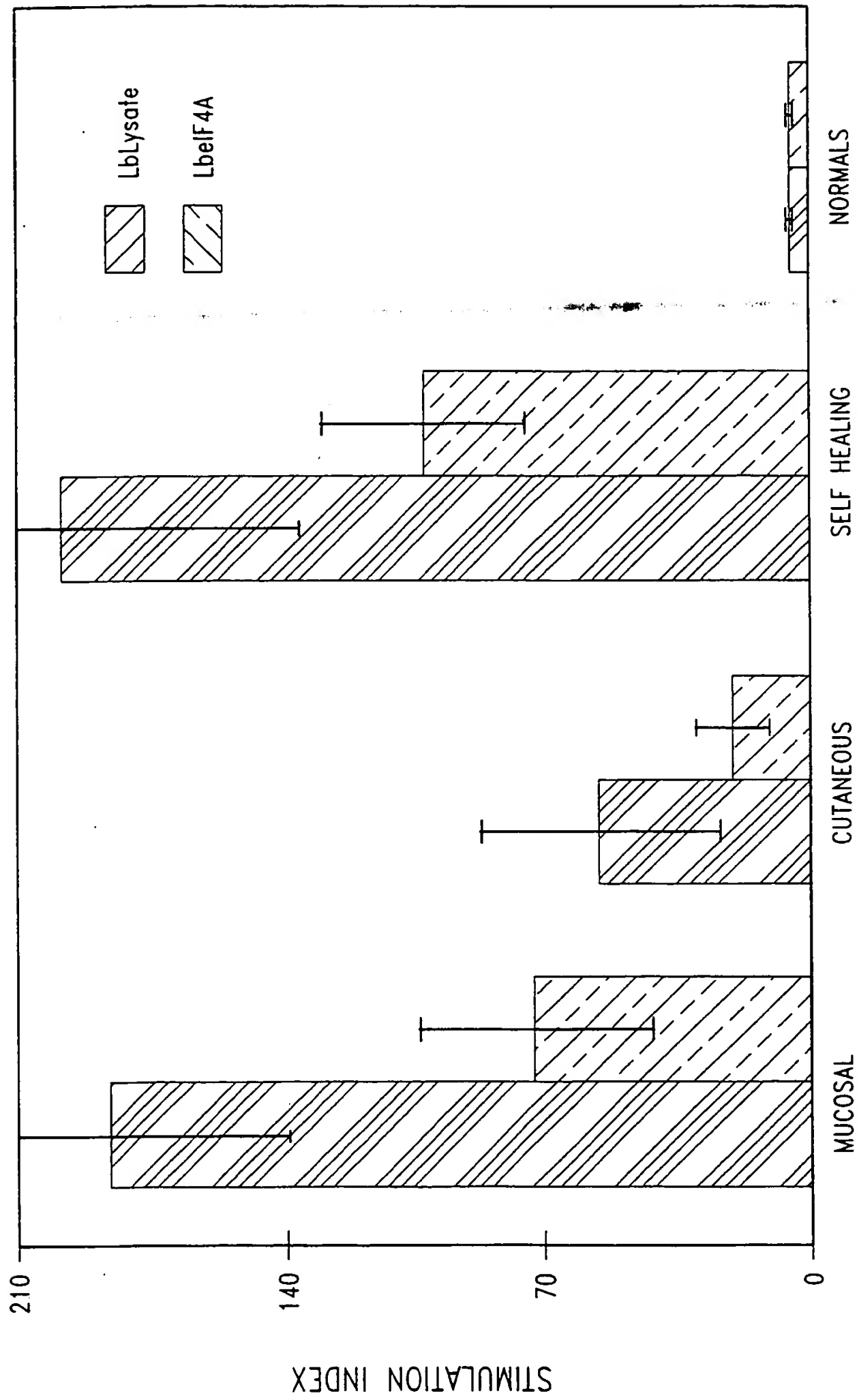


Fig. 3



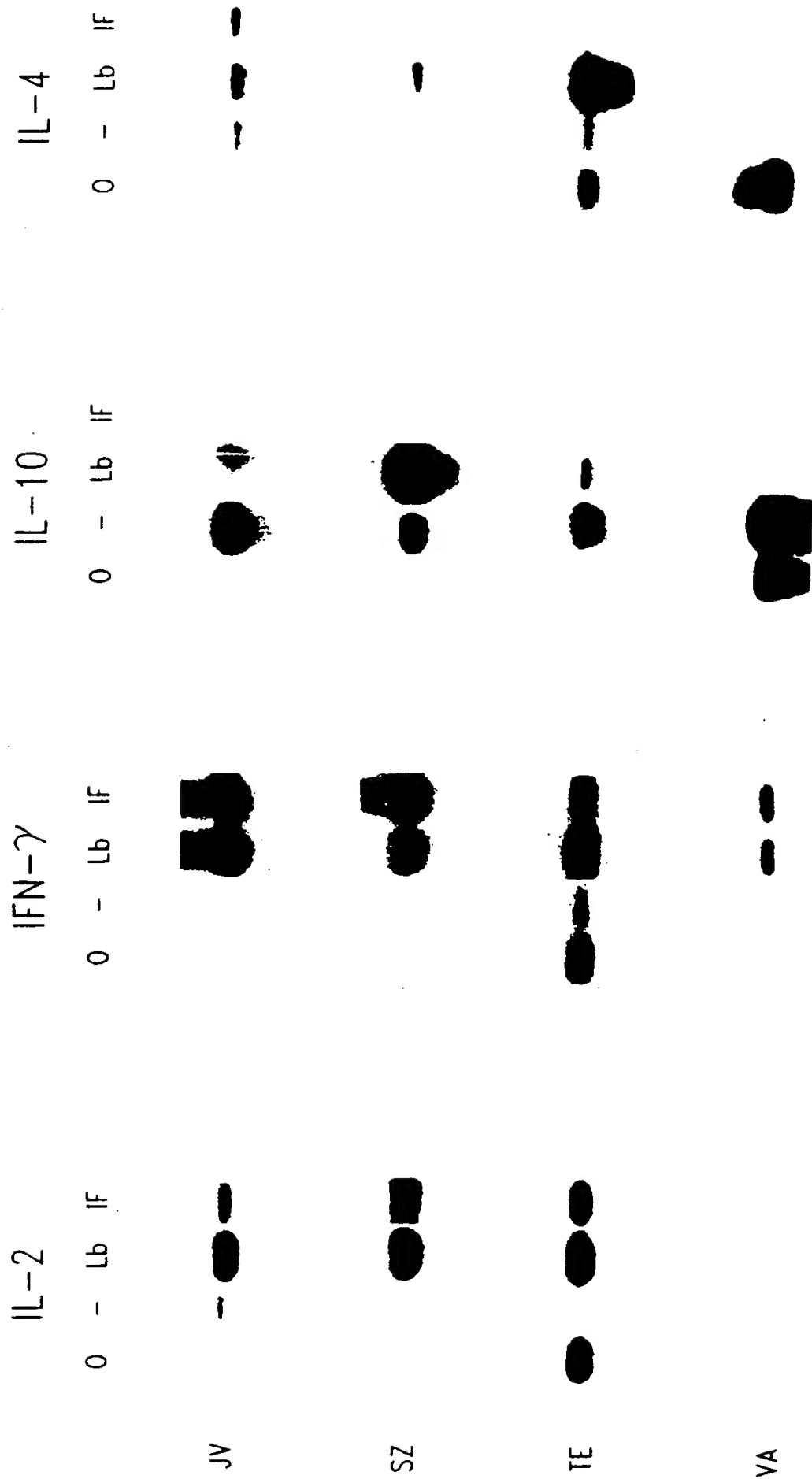


Fig. 4A

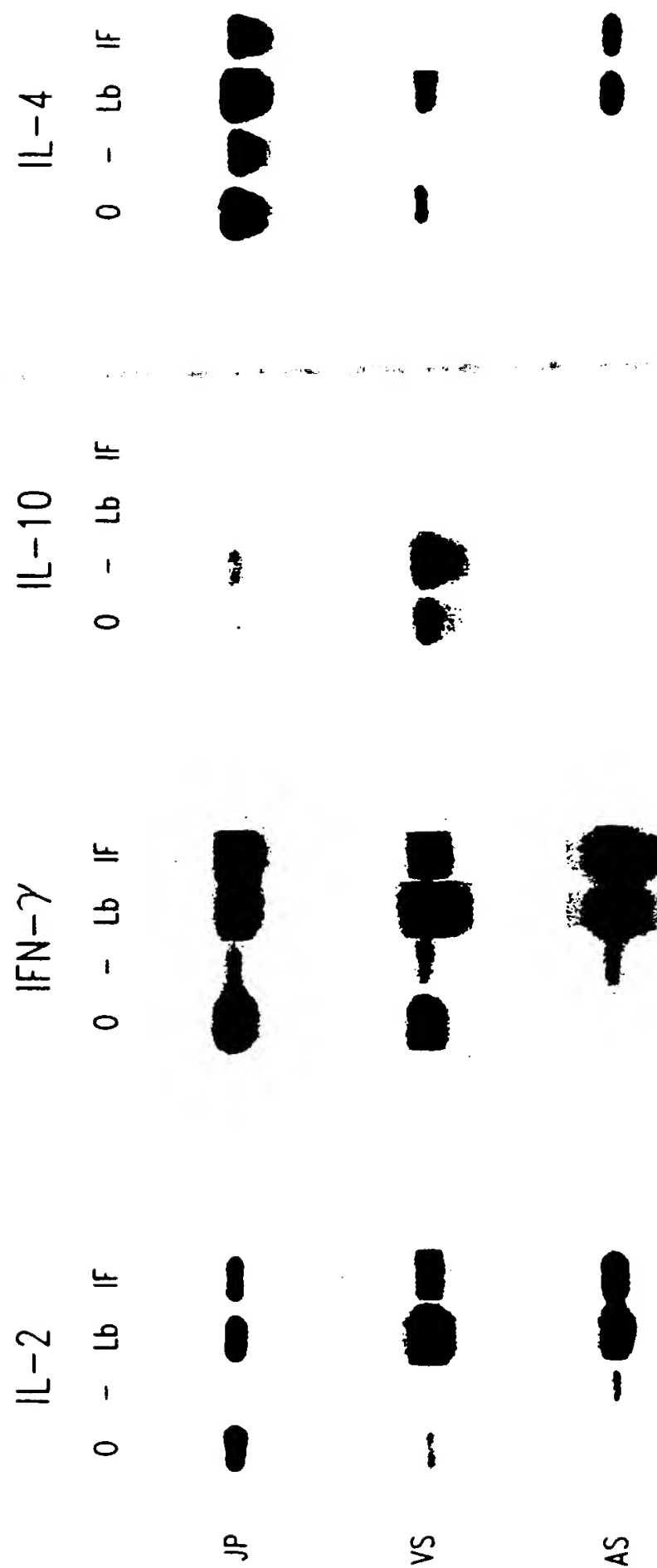


Fig. 4B

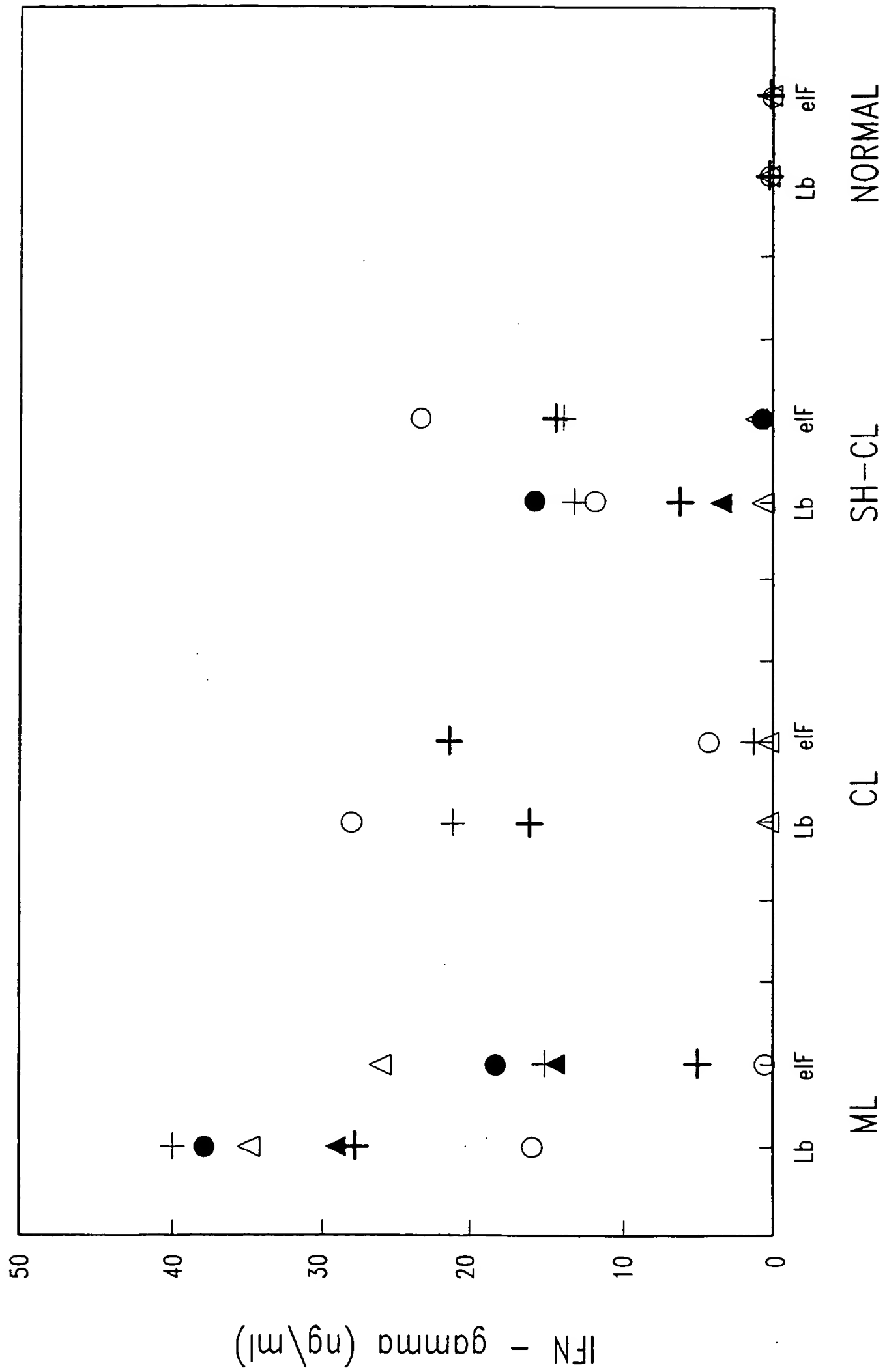


Fig. 5

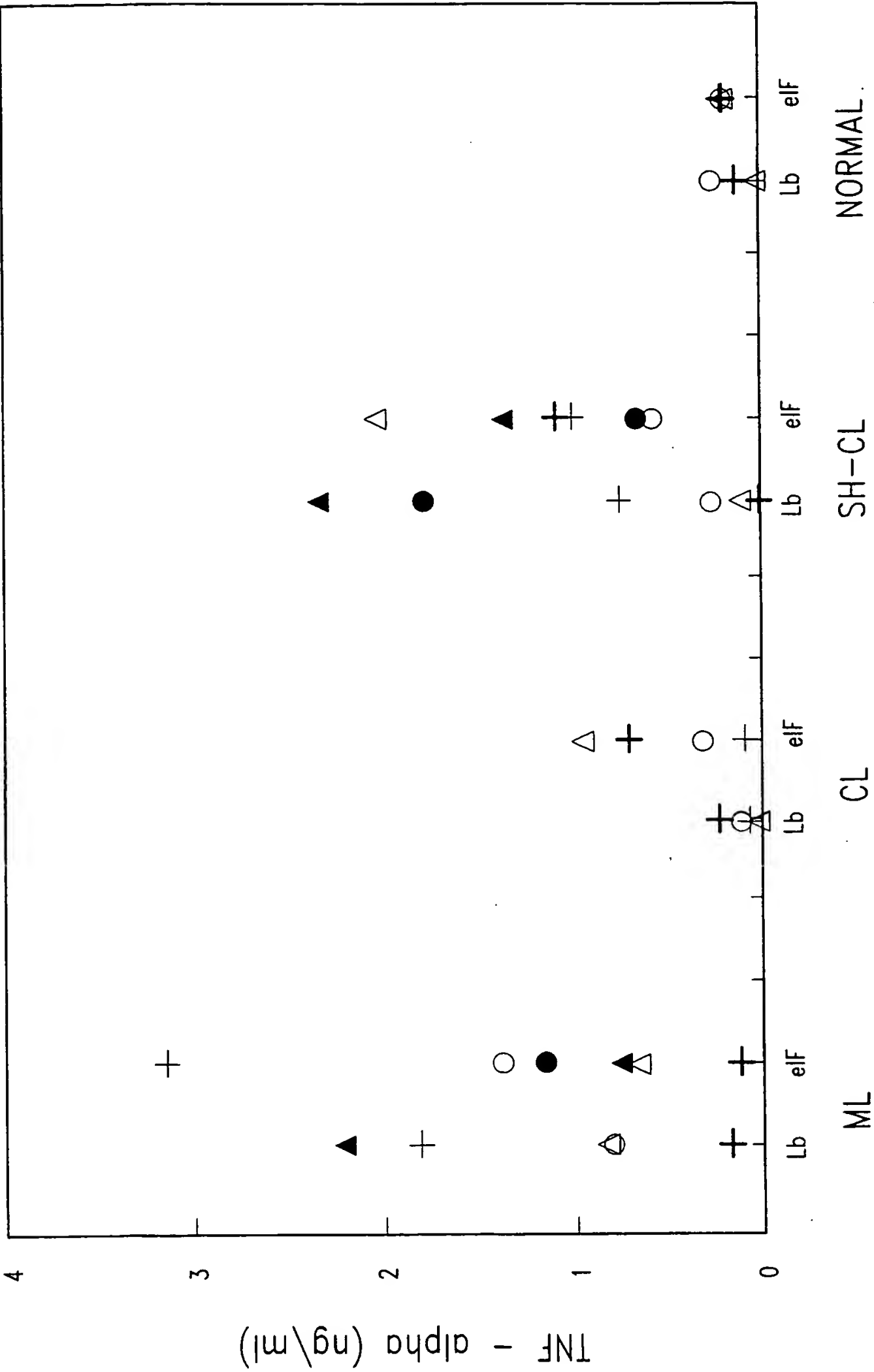
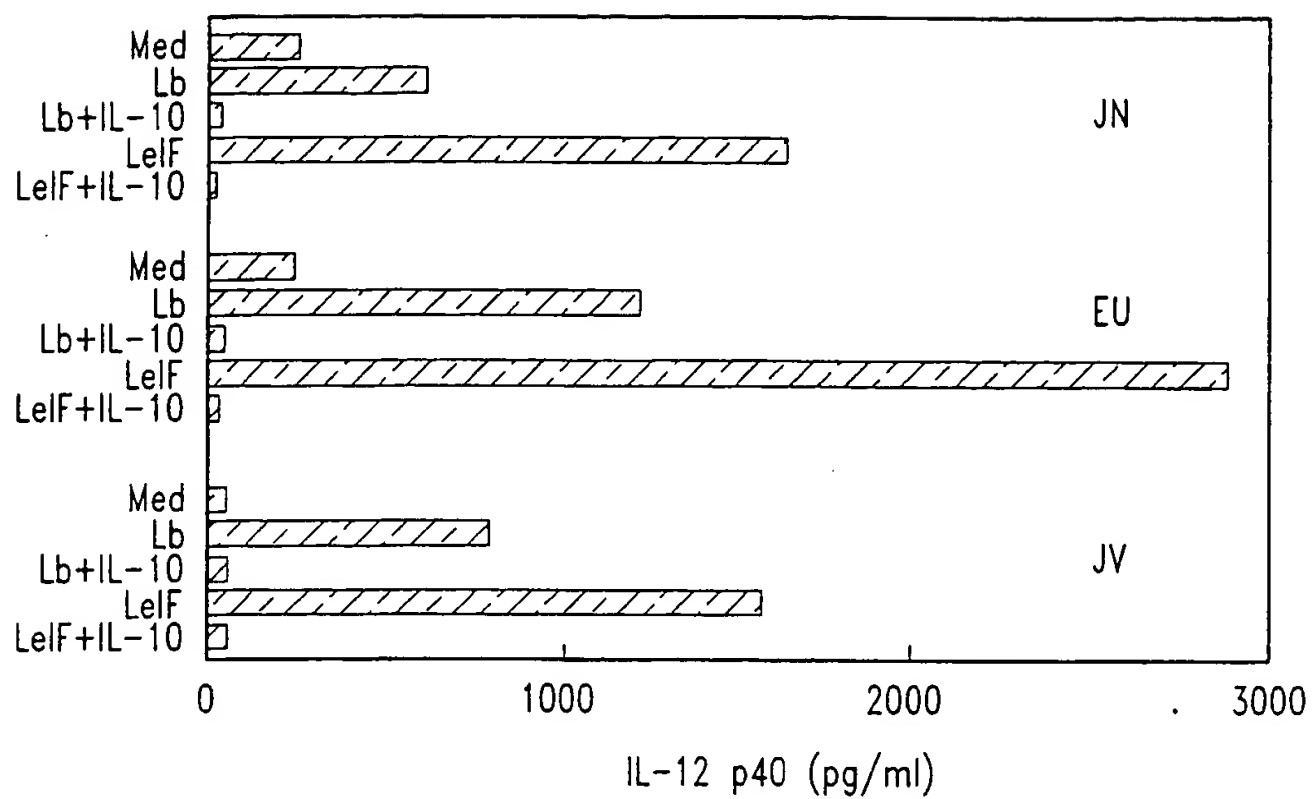
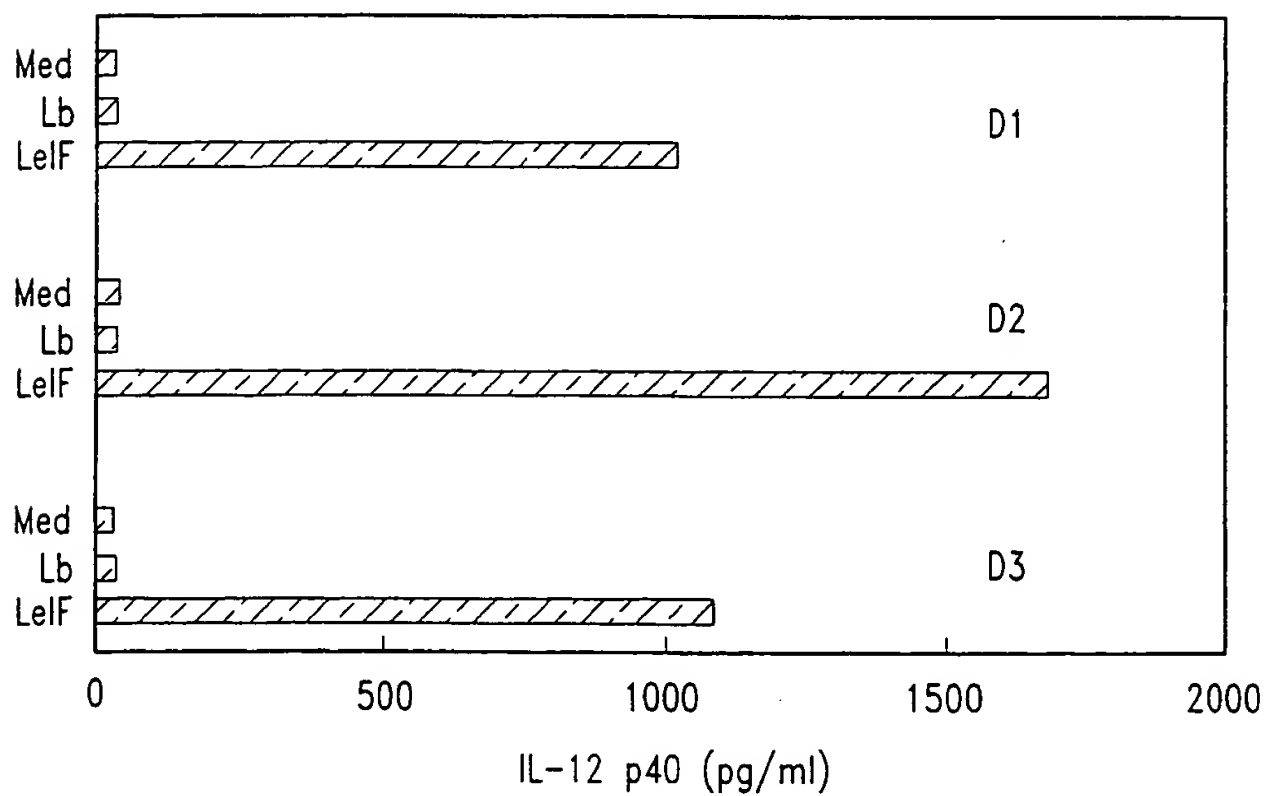
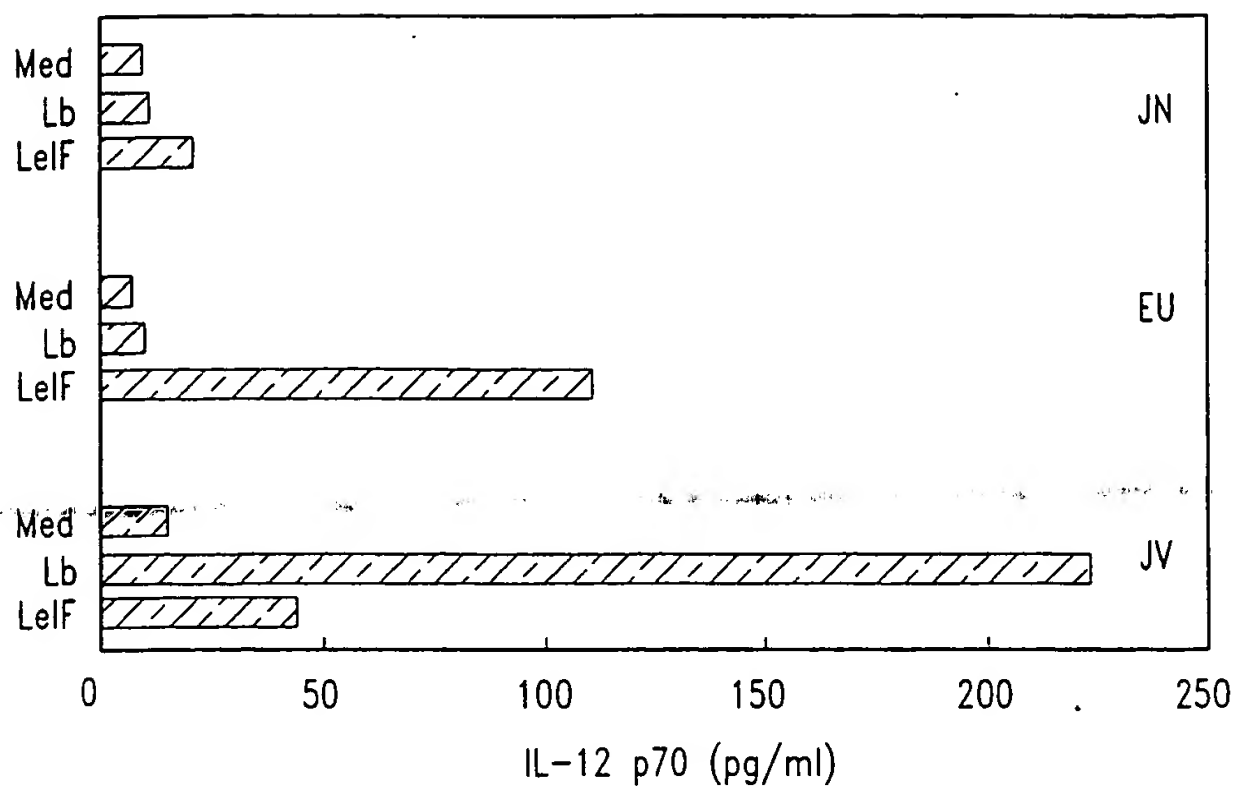
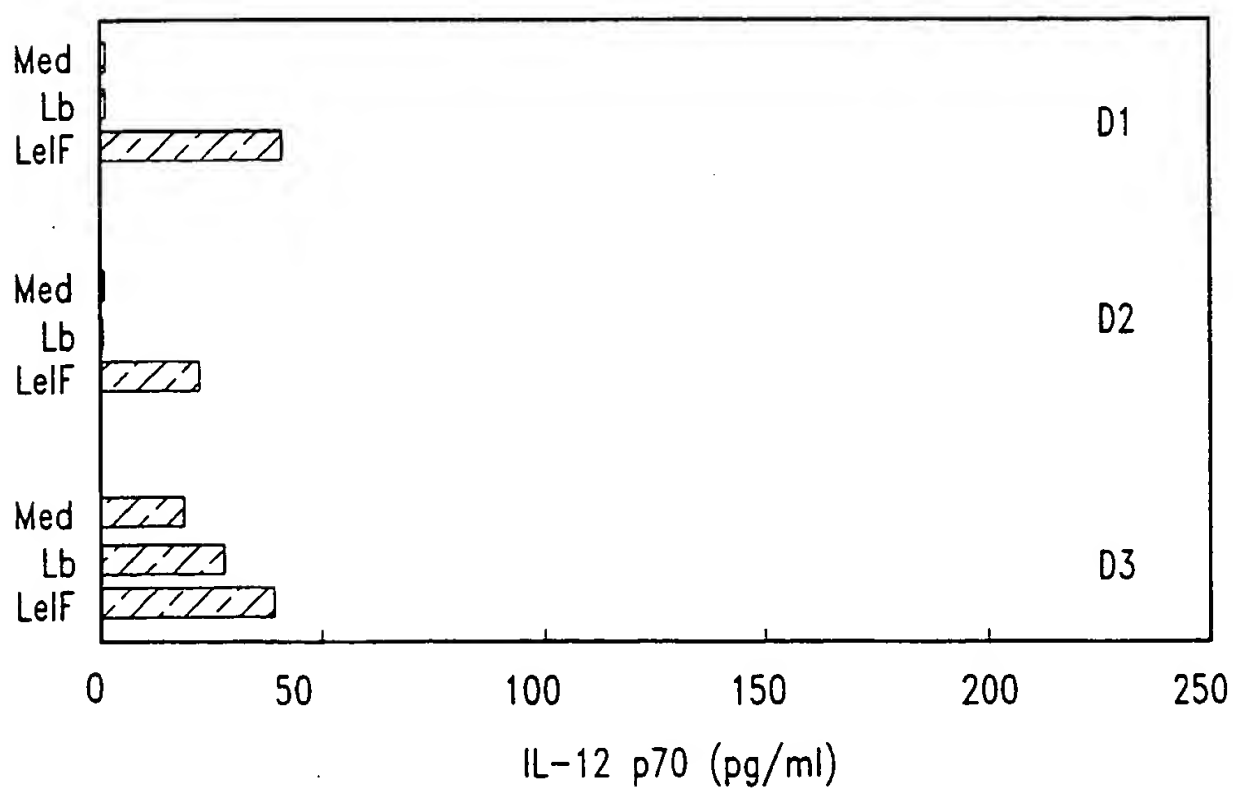


Fig. 6

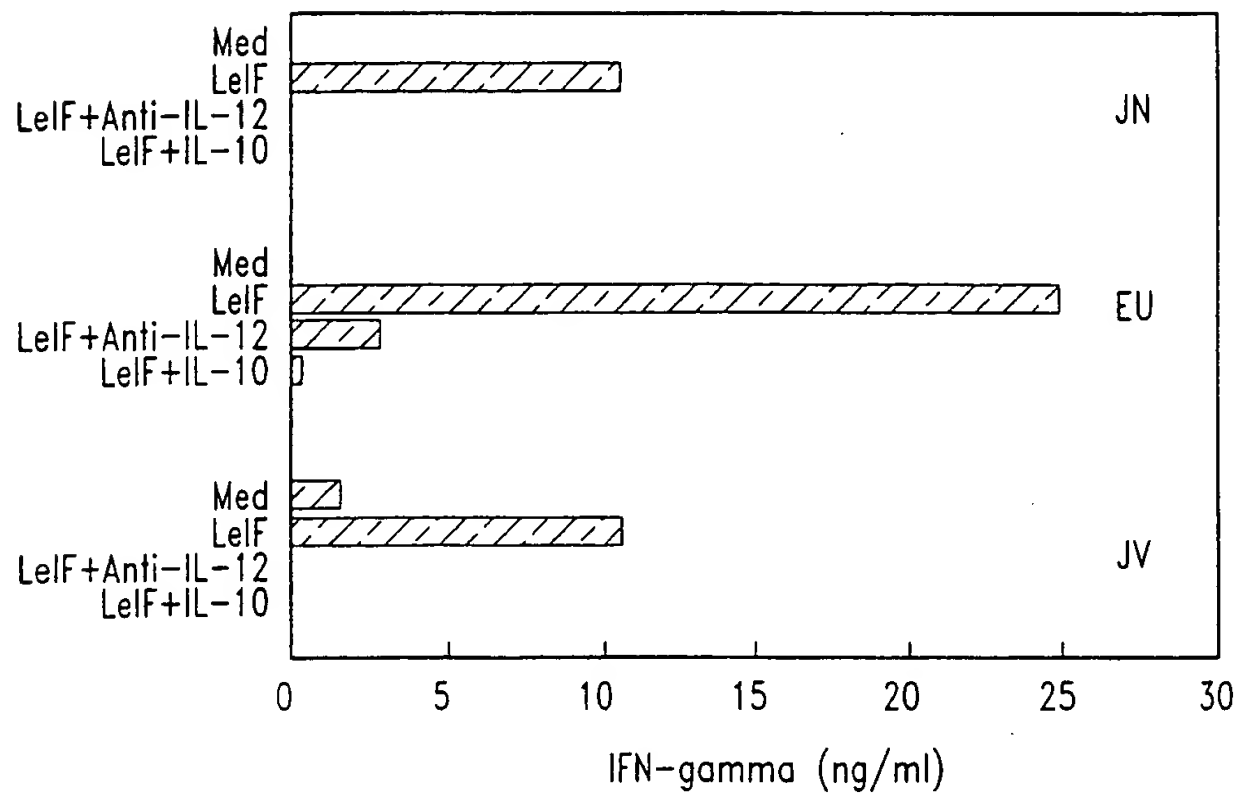
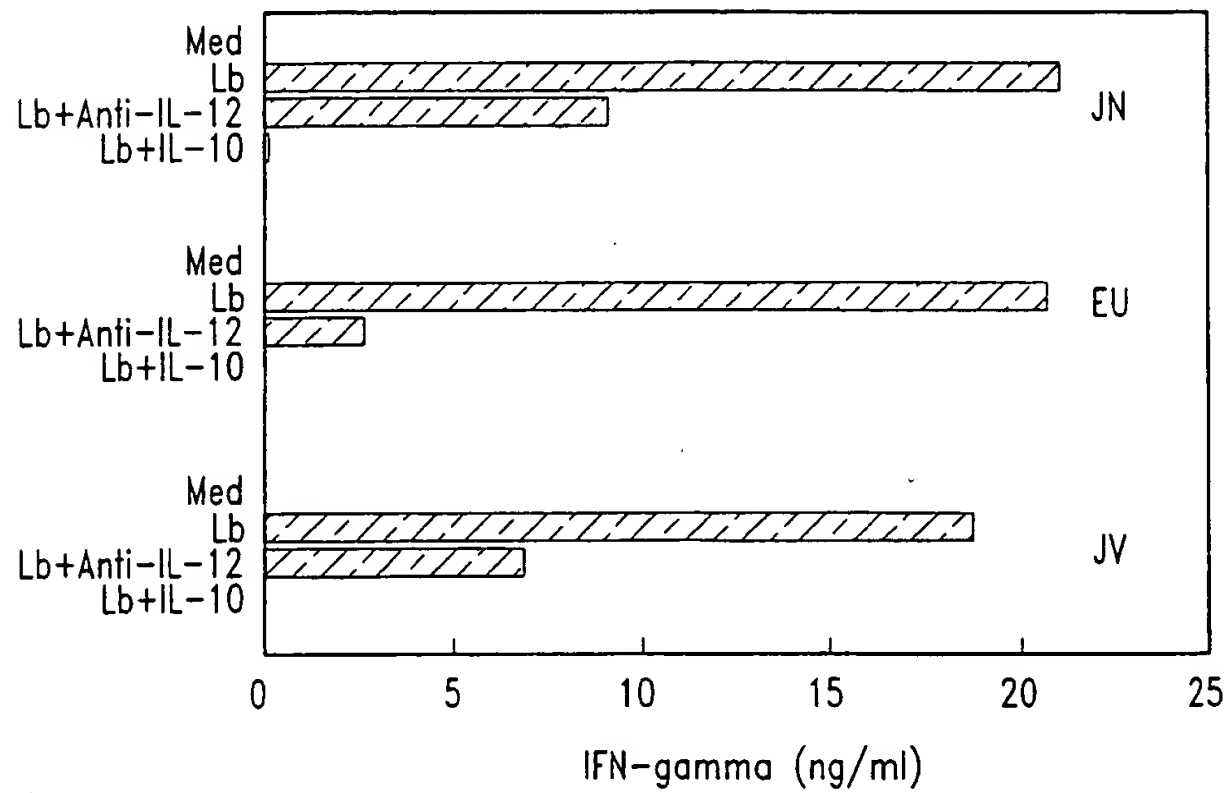
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*Fig. 7A**Fig. 7B*

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*Fig. 7C**Fig. 7D*

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*Fig. 8A**Fig. 8B*

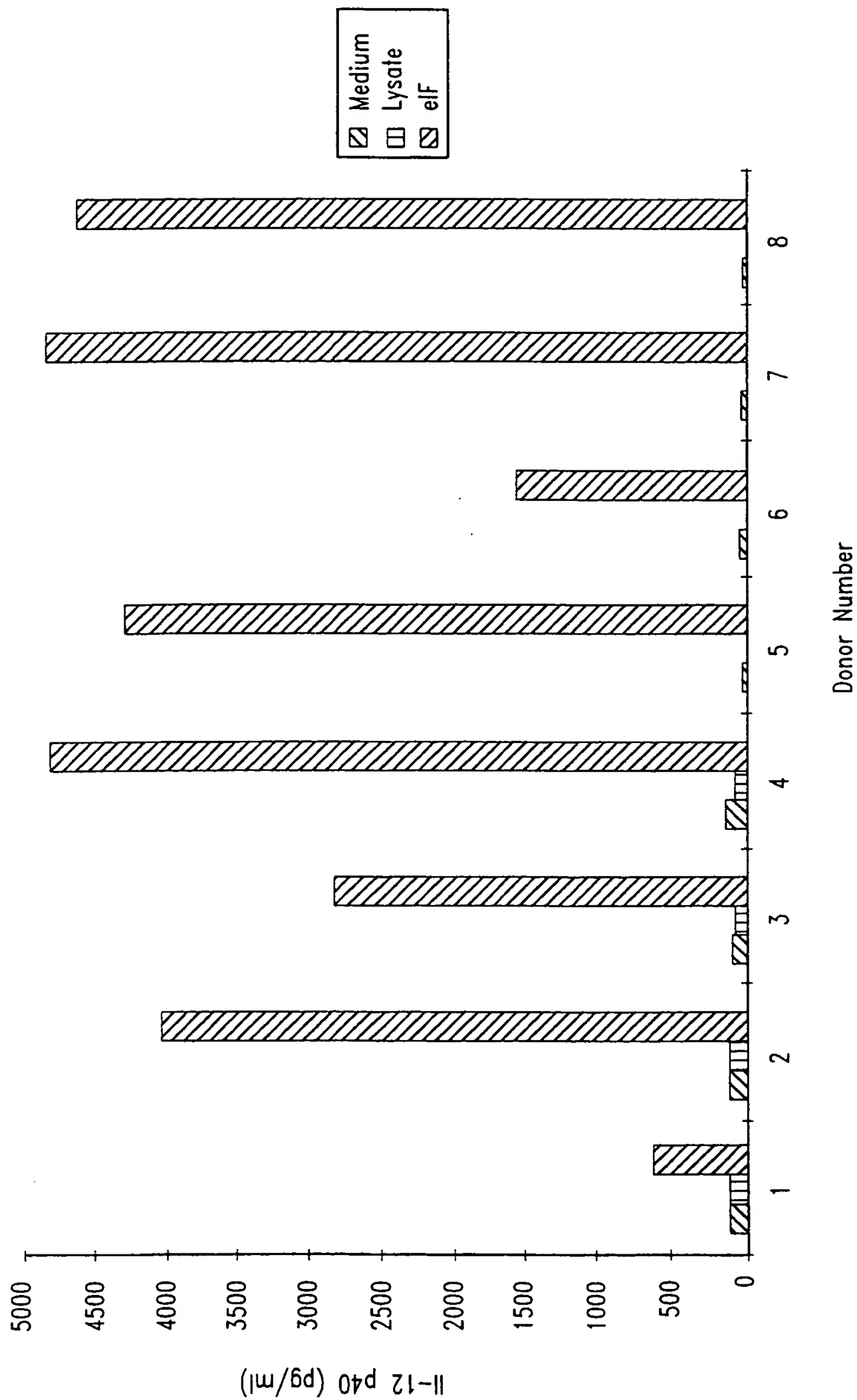
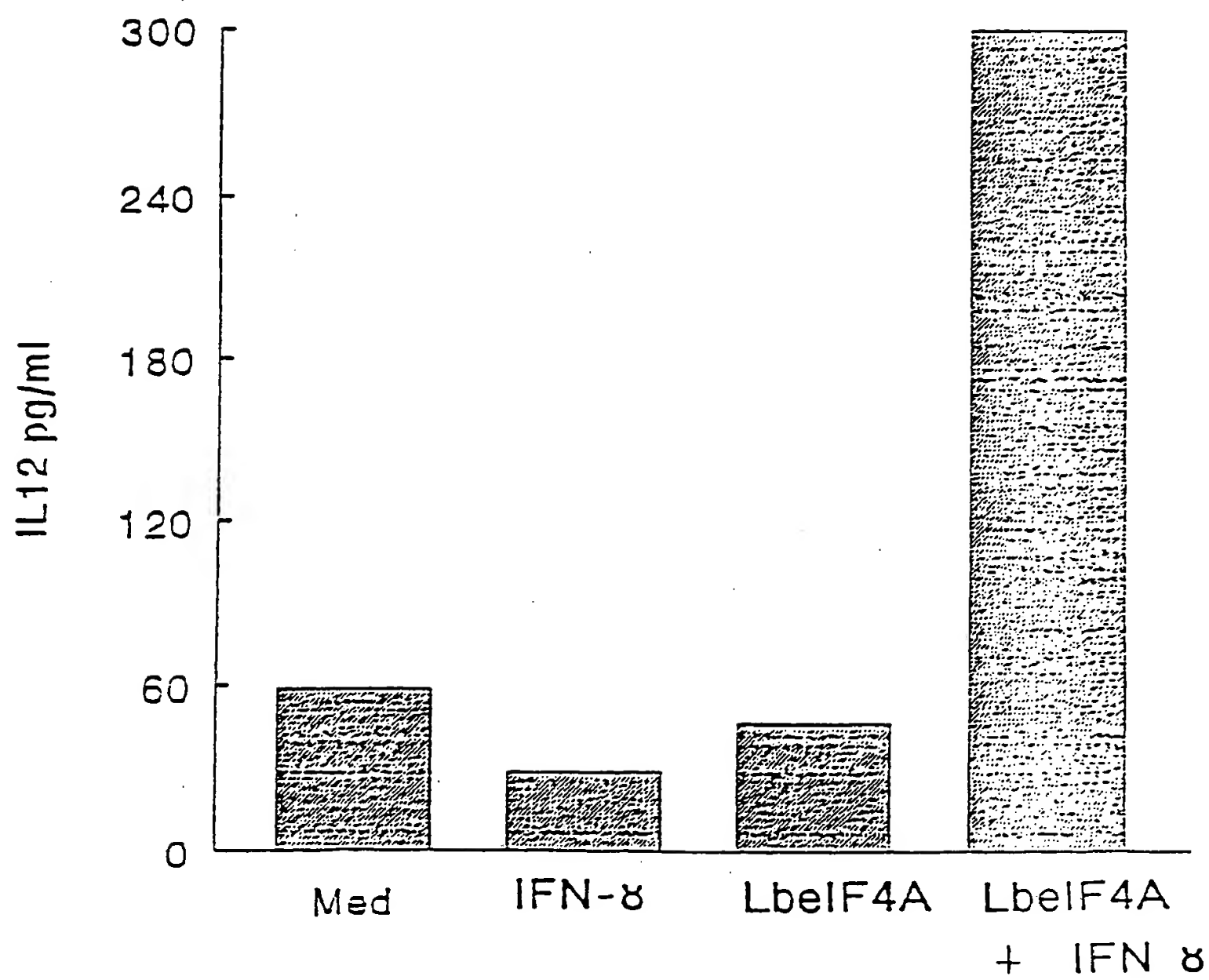


Fig. 9A



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## IL12 Induction

**FIG. 9B**

IL-12 p40 Production by THP-1  
Cells With eIF and IFN-gamma

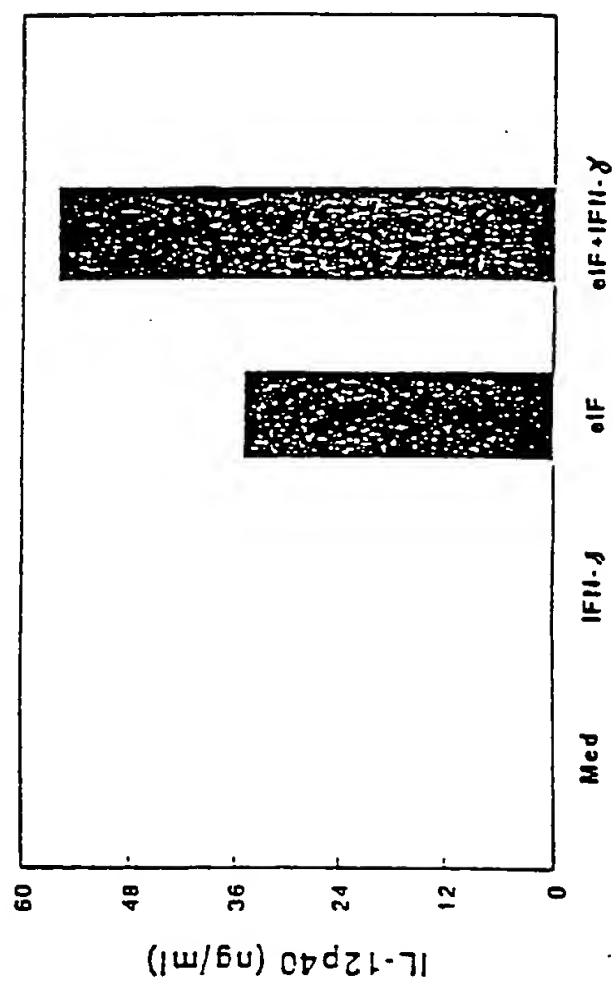


FIG. 10

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# Cytokine Production by Lymph Node Cells Primed With Lysate or Recombinant Lbelf4A

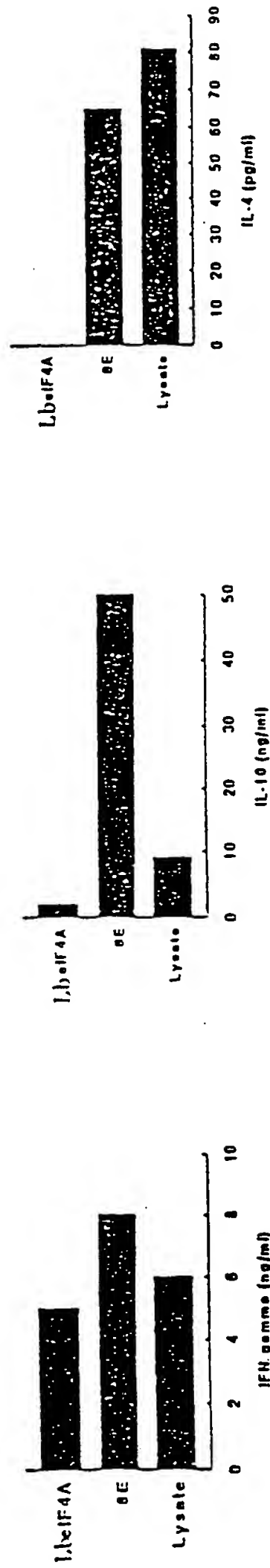


FIG. 11

# Protection of BALB/c Mice Against L. major Infection With LbelF4A

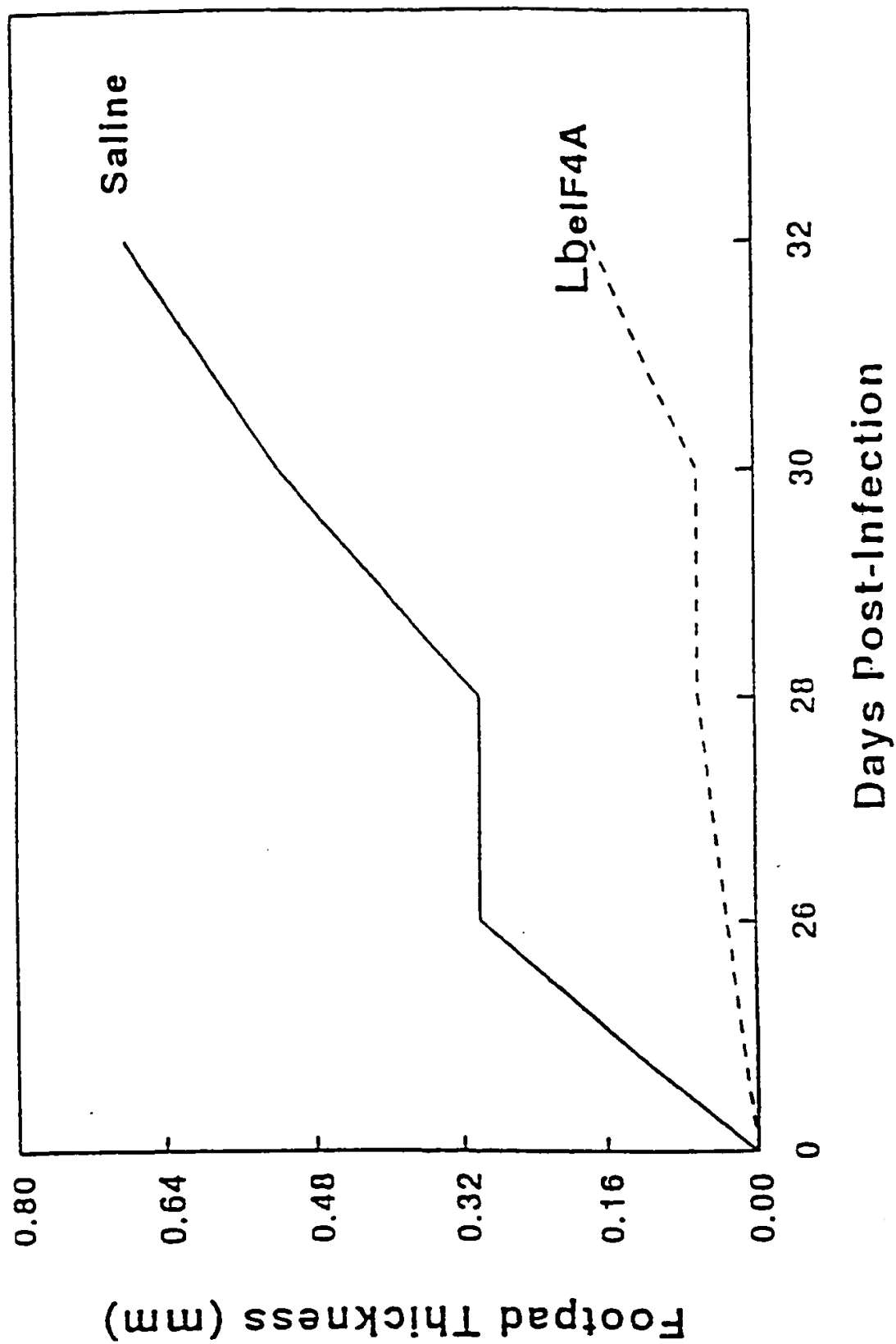
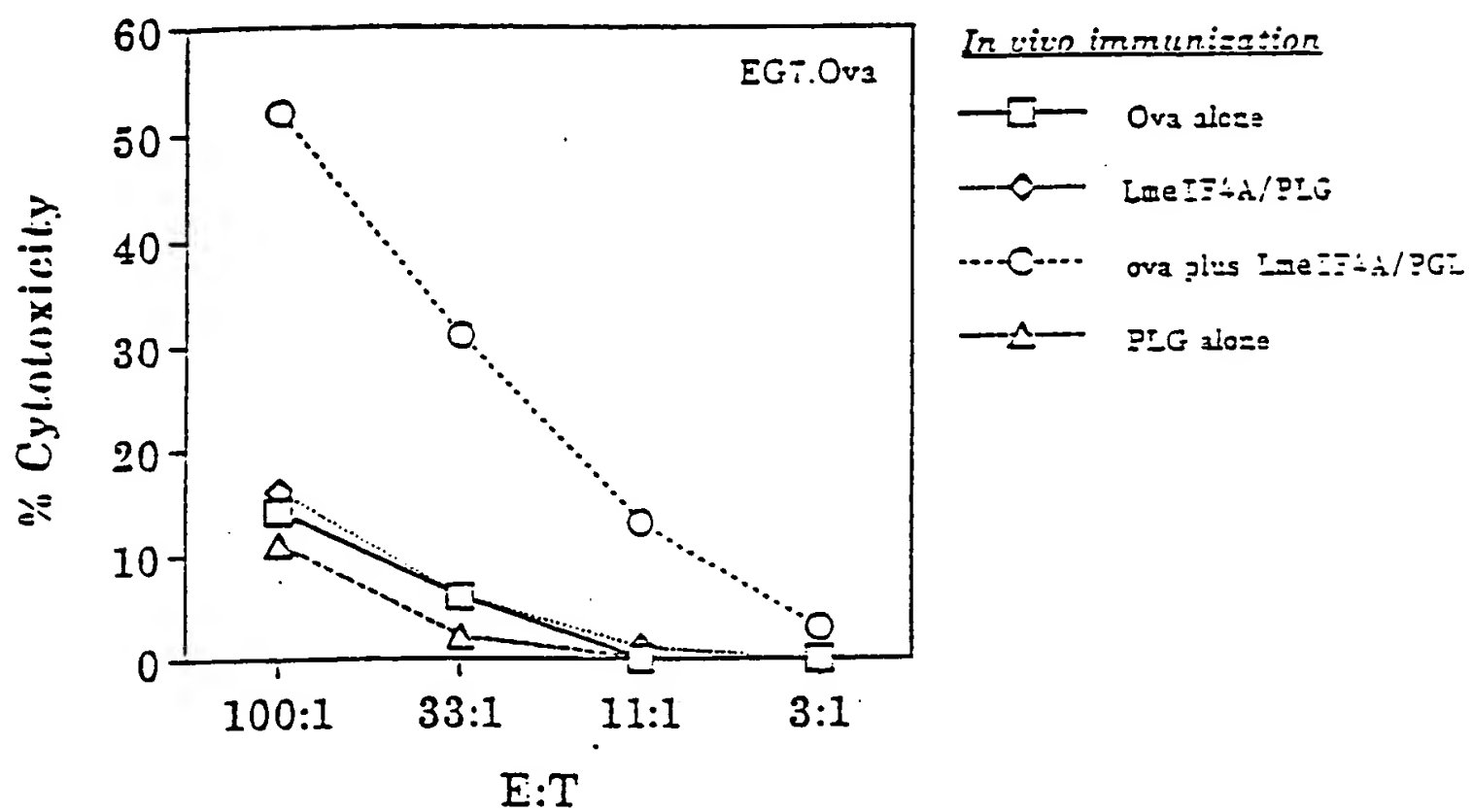


FIG. 12

### *Anti-Ova CTL Elicitation Using LmeIF4A*



**FIG. 13**

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# Lmelf4A as Adjuvant for the Induction of TNP-Specific Antibodies

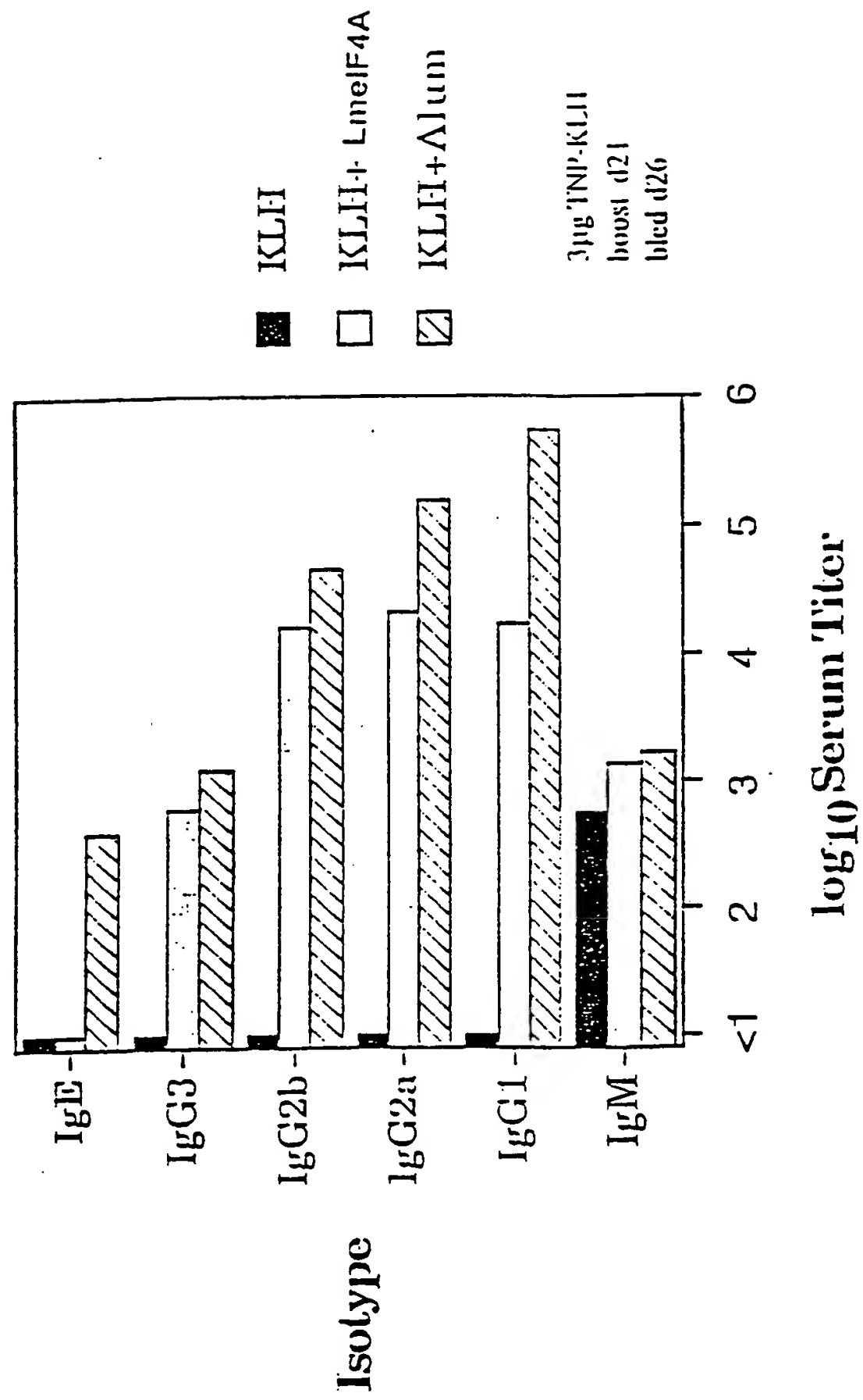
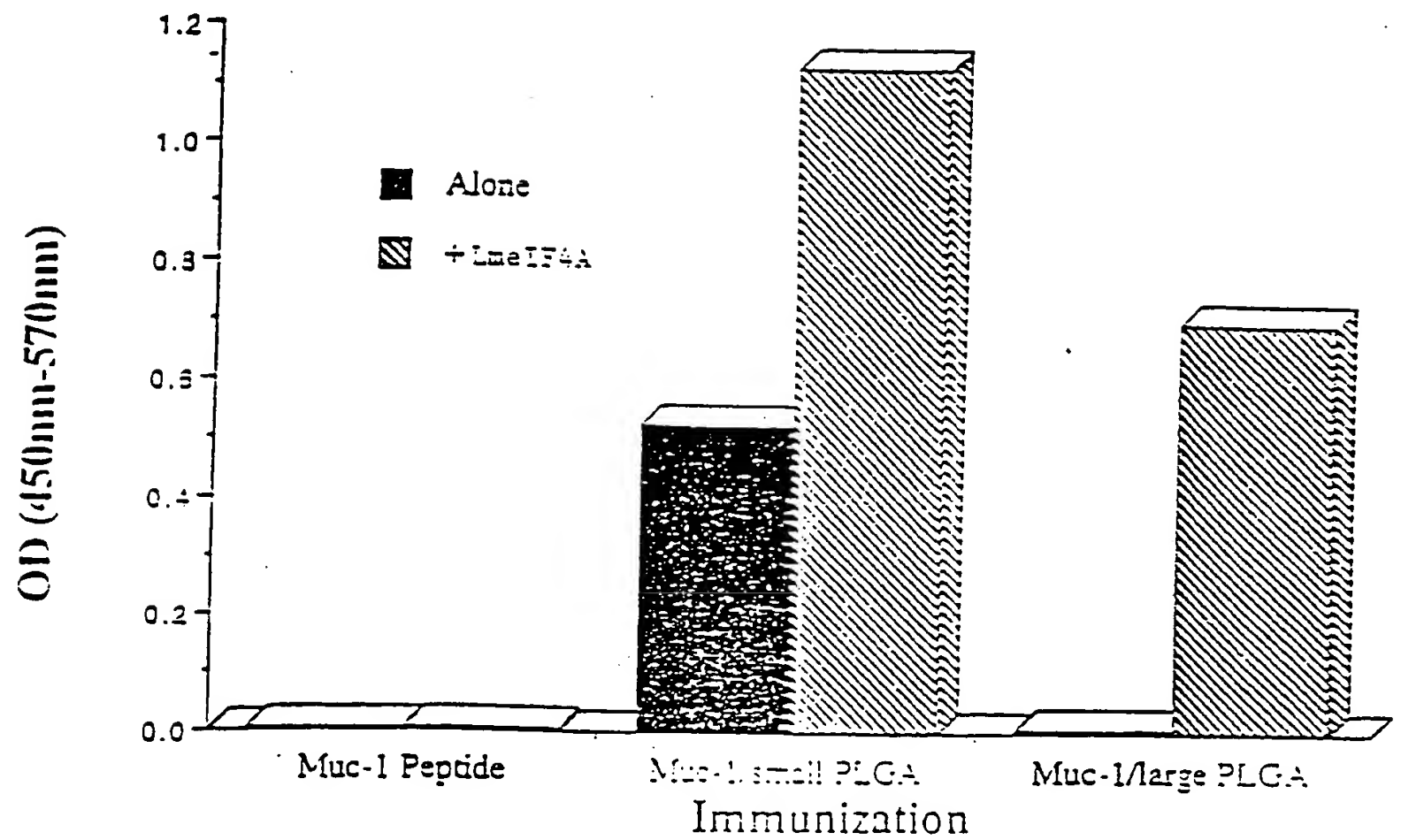


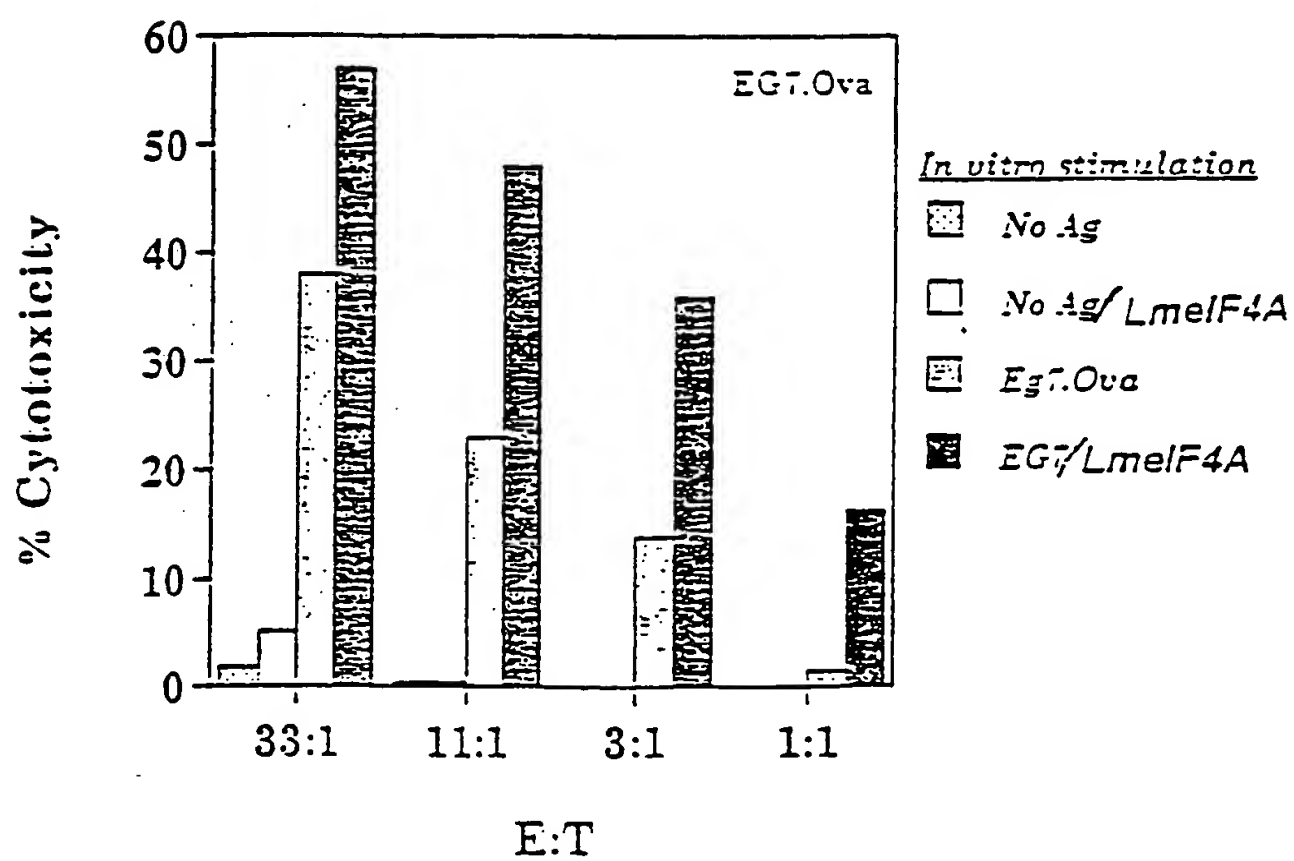
FIG. 14

### LmeIF4A Enhances anti-Muc-1 Antibody Production in Mice



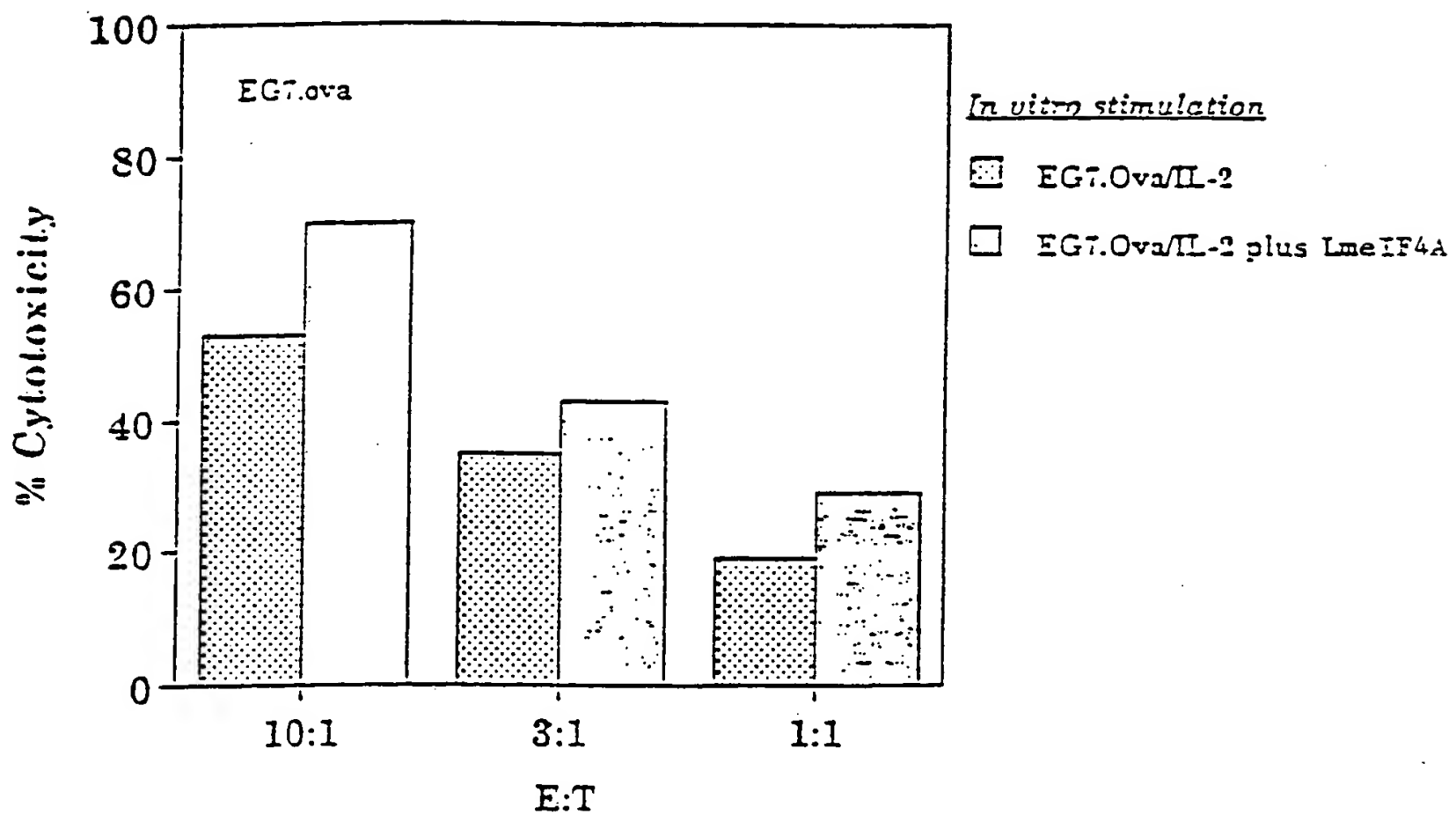
**FIG. 15**

# *Anti-Ova CTL Induction Using Ova/PLG*



**FIG. 16**



*LmeIF4A Enhances CTL Activities ex vivo***FIG. 17**

## Induction of Murine Alloreactive CTL by Lmelf4A

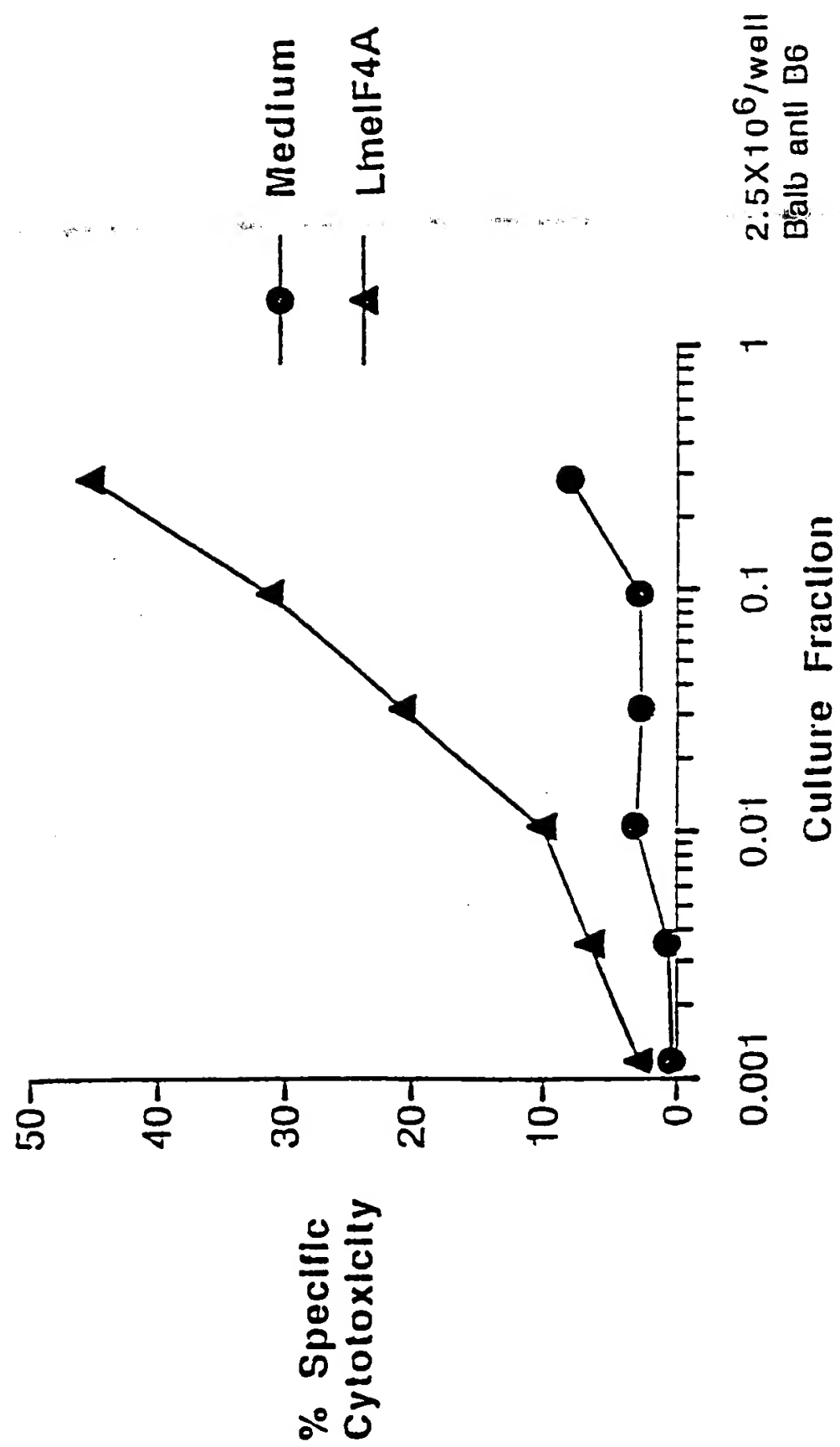


FIG. 18

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Adjuvant Effect of LeIF in N1 Tumor Therapy

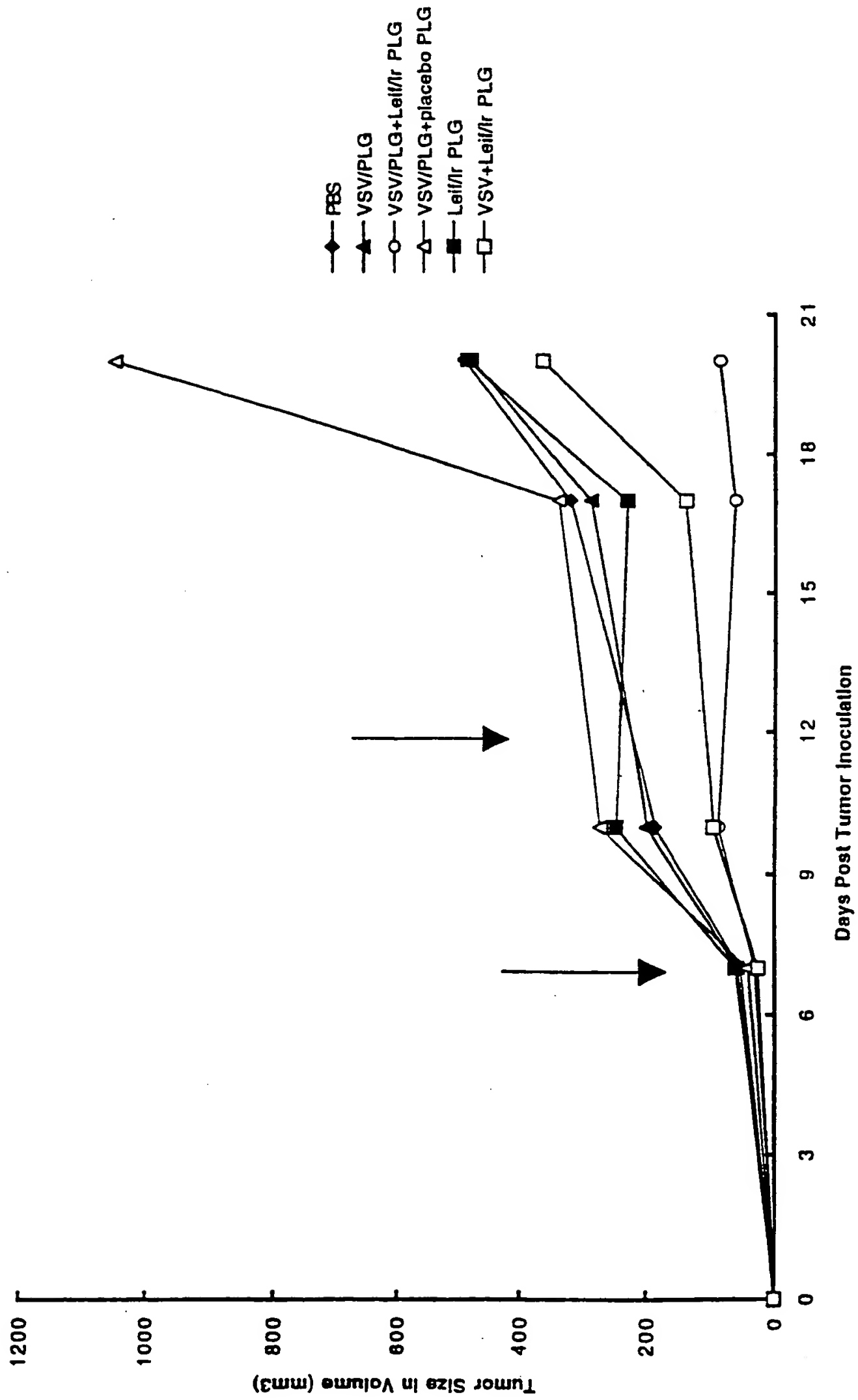


FIG. 19

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Adjuvant Effect of LeiF in N1 Tumor Therapy

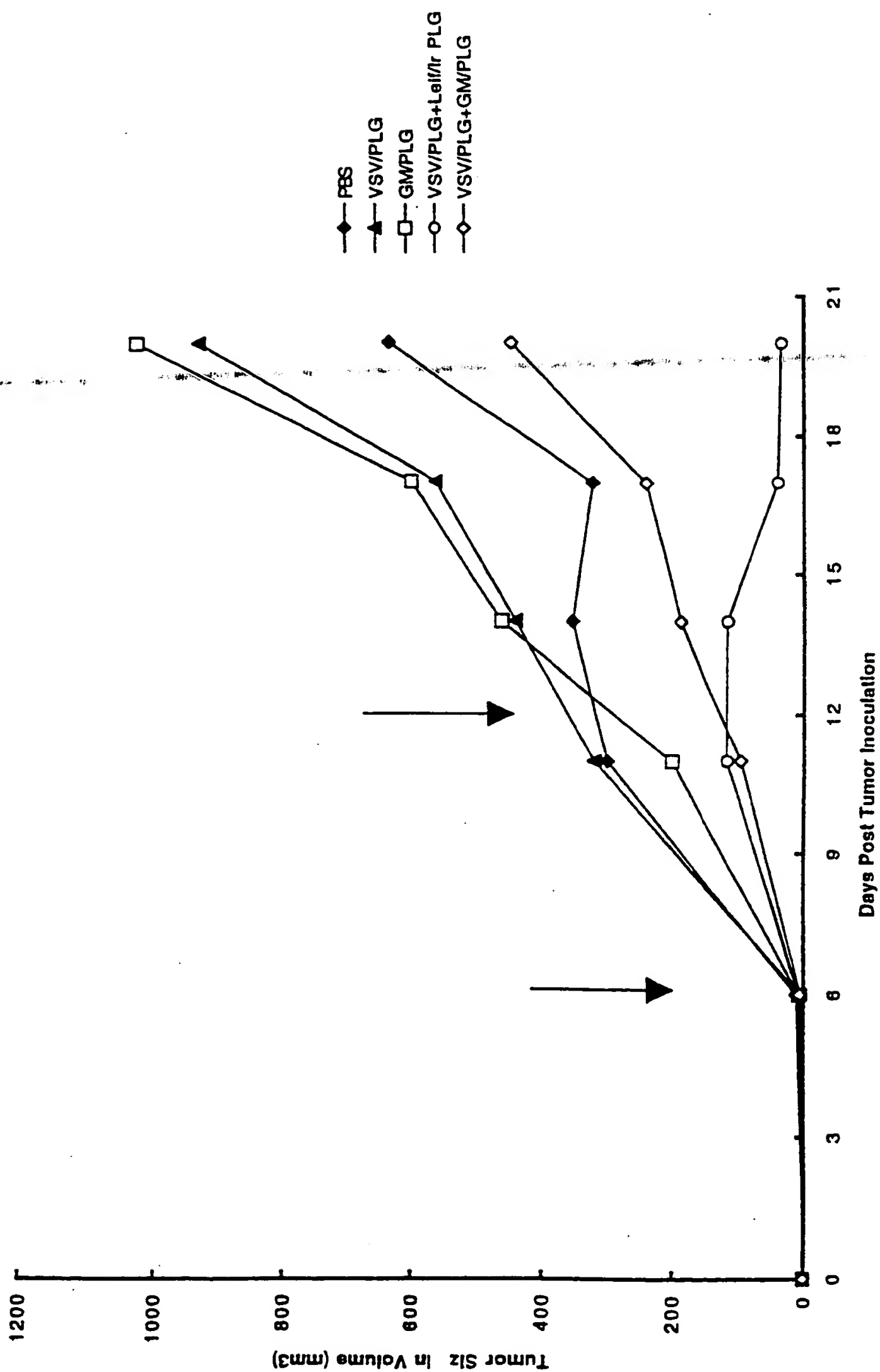


FIG. 20

Adjuvant Effect of LeIF in N1 Tumor Therapy

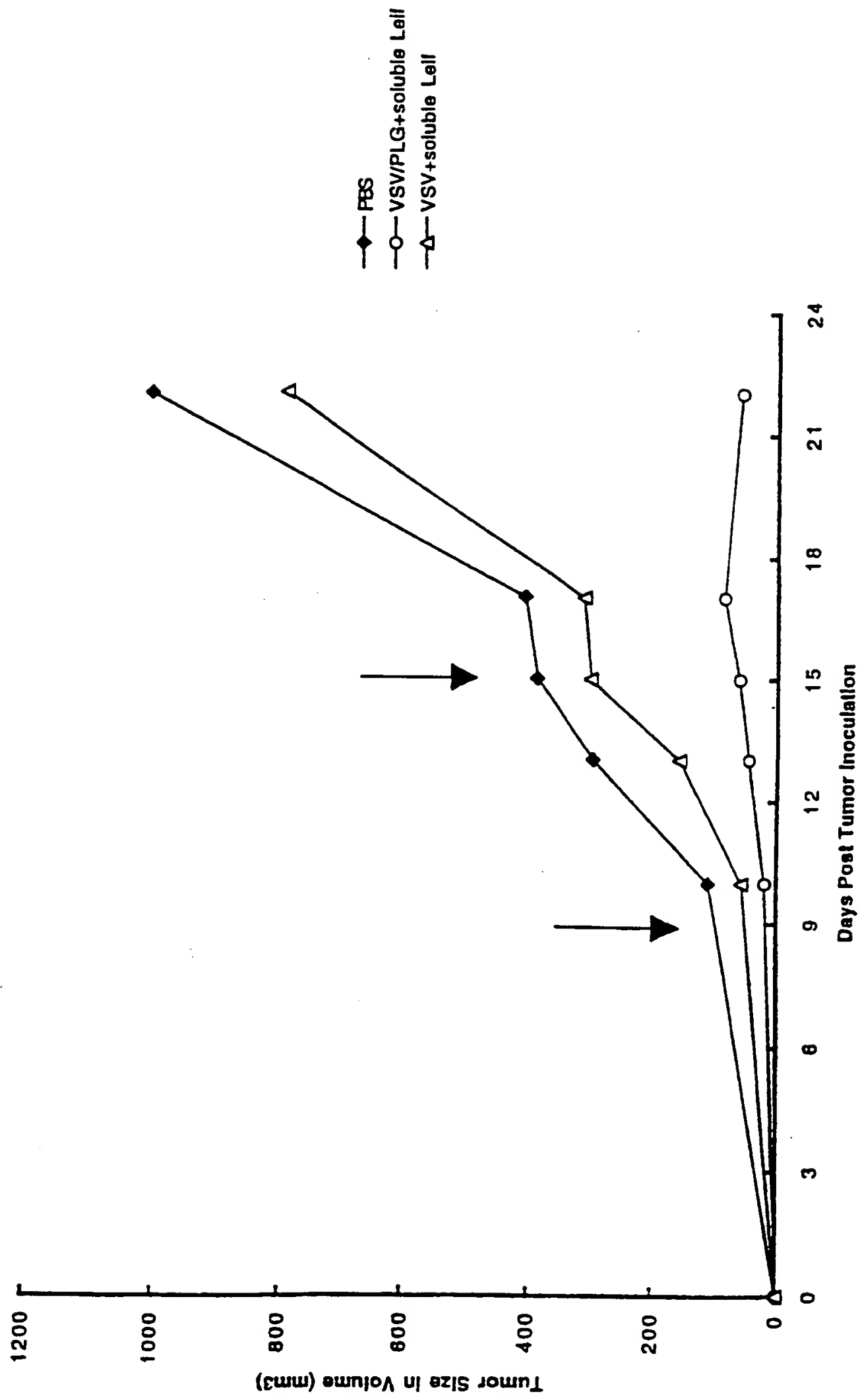


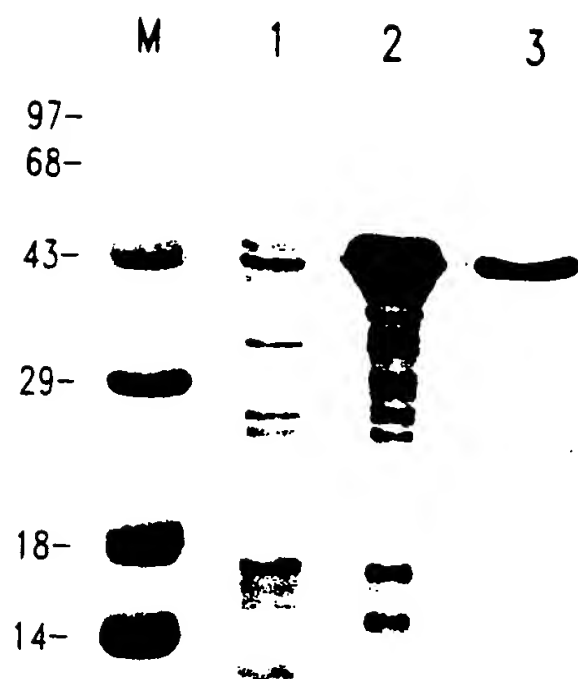
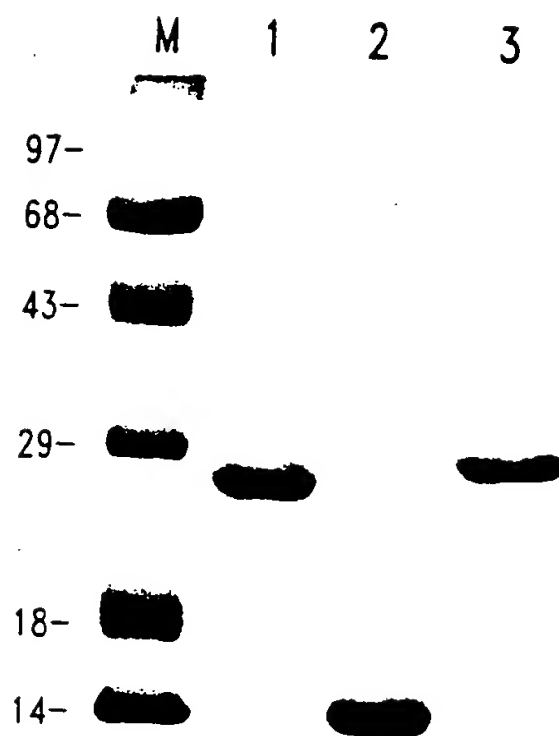
FIG. 21

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MAQNDKIAPQDQDSF--LDDQPGVRP-----IPSFDDMPLHQNLLRGIYS	LmeIF
MSQQDRVAPQDQDSF--LDDQPGVRP-----IPSFDDMPLHQNLLRGIYS	LbeIF
MSGGSADYNREHGGPEGMDPDGVIESNWEIVDNFDDMNLKESLLRGIYA	MueIF
MSGGSADYNREHGGPEGMDPDGVIESNWEIVDNFDDMNLKESLLRGIYA	HueIF
YGFEKPSSIQQRAIAPFTRGGDIIAQASGTGKTGAFSIGLLQRLDFRHN	LmeIF
YGFEKPSSIQQRAIAPFTRGGDIIAQASGTGKTGAFSIGLLQRLDFRHN	LbeIF
YGFEKPSAIIQRAIIPCICKGYDVIAQAQSGTGKTATFAISILQQLEIEFK	MueIF
YGFEKPSAIIQRAIIPCICKGYDVIAQAQSGTGKTATFAISILQQLEIEFK	HueIF
<div style="text-align: center;"> <math>\leftarrow</math> A-motif <math>\rightarrow</math> </div>	
LIQGLVLSPTRELALQTAEVISRIGEFLSNSSKFCETFVGGTRVQDDLRLK	LmeIF
LIQGLVLSPTRELALQTAEVISRIGEFLSNSAKFCETFVGGTRVQDDLRLK	LbeIF
ETQALVLAPTRELAQQIQKVILALGDYMGAT--CHACIGGTNVRNEMQK	MueIF
ETQALVLAPTRELAQQIQKVILALGDYMGAT--CHACIGGTNVRNEMQK	HueIF
<div style="text-align: center;"> <math>\leftarrow</math> B-motif II <math>\rightarrow</math> </div>	
LQAGVI-VAVGTPGRVSDVIKRGALRTESLRVLVLDEADEMLSQGFADQI	LmeIF
LQAGVM-VAVGTPGRVSDVIKRGALRTESLRVLVLDEADEMLSQGFADQI	LbeIF
LQAEAPHIVVGTPGRVFDMLNRRYLSPKWKMFVLDEADEMLSRGFKDQI	MueIF
LQAEAPHIVVGTPGRVFDMLNRRYLSPKWKMFVLDEADEMLSRGFKDQI	HueIF
YEIFRFLPKDIQVALFSATMPEEVLELTKKFMRDPVRILVKRESLTLEGI	LmeIF
YEIFRFLPKDIQVALFSATMPEEVLELTKKFMRDPVRILVKRESLTLEGI	LbeIF
YERVQKLNTSIQVVLLSATMPTDVLEVTKKFMRDPTRILVKKEELTLEGI	MueIF
YEIFQKLNTSIQVVFASATMPTDVLEVTKKFMRDPTRILVKKEELTLEGI	HueIF
KQFFIAVE-EEHKLDTLMDLYETVVSIAQSVIFANTRRKVDWIAEKLNQSN	LmeIF
KQFFIAVE-EEHKLDTLMDLYETVVSIAQSVIFANTRRKVDWIAEKLNQSN	LbeIF
KQFYIINVEREEWKLDTLCDLYETLTITQAVIFLNTRRKVDWLTEKMQAIY	MueIF
KQFYIINVEREEWKLDTLCDLYETLTITQAVIFLNTRRKVDWLTEKMHARD	HueIF
HTVSSMHAEMPKSDRERVMNTFRSGSSRVLVTDDLVARGIDVHHVNIVIN	LmeIF
HTVSSMHAEMPKSDRERVMNTFRSGSSRVLVTDDLVARGIDVHHVNIVIN	LbeIF
FTVSAHGDMDQKERDVIIMREFRSGSSRVLITTDLLARGIDVQQVSLVIN	MueIF
FTVSAHGDMDQKERDVIIMREFRSGSSRVLITTDLLARGIDVQQVSLVIN	HueIF
FDLPTNKENYLHRIGRGGRYGRKGVAINFVTEKDVELLHEIEAHYHTQID	LmeIF
FDLPTNKENYLHRIGRGGRYGVKGVAINFVTEKDVELLHEIEGHYHTQID	LbeIF
YDLPTNRENYIHRIGRGGRFGRKGVAINFVTEEDKRILRHIETFYNTTVE	MueIF
YDLPTNRENYIHRIGRGGRFGRKGVAINFVTEEDKRILRDIETFYNTTVE	HueIF
ELPVDFAAAYLGE	LmeIF
ELPVDFAAAYLGE	LbeIF
EMPMNGADLI	MueIF
EMPMNVADLI	HueIF

Fig. 22

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*Fig. 23A**Fig. 23B*

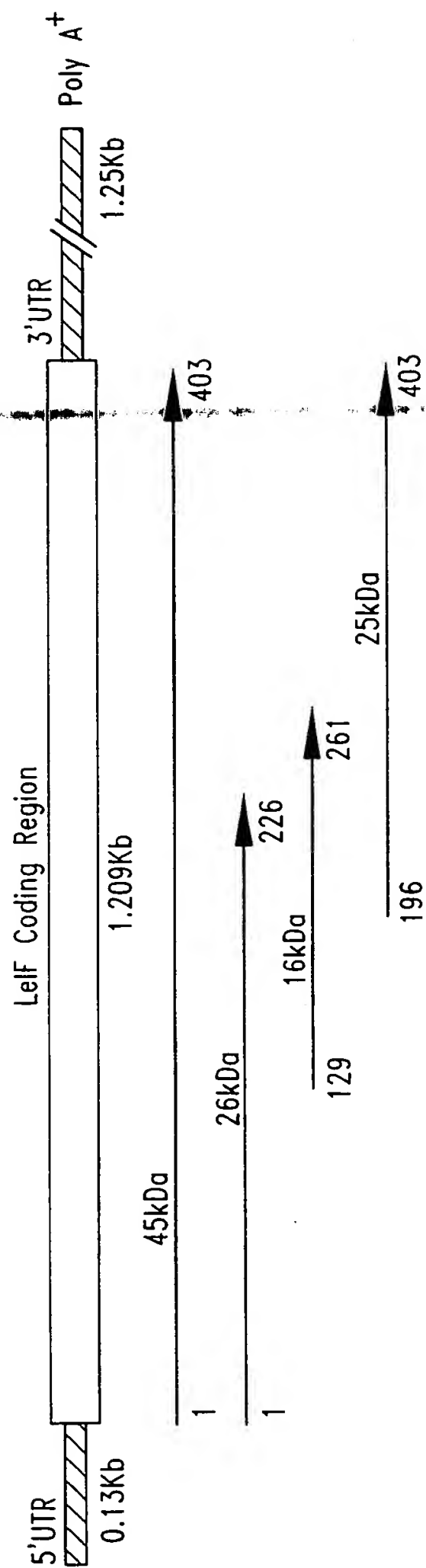
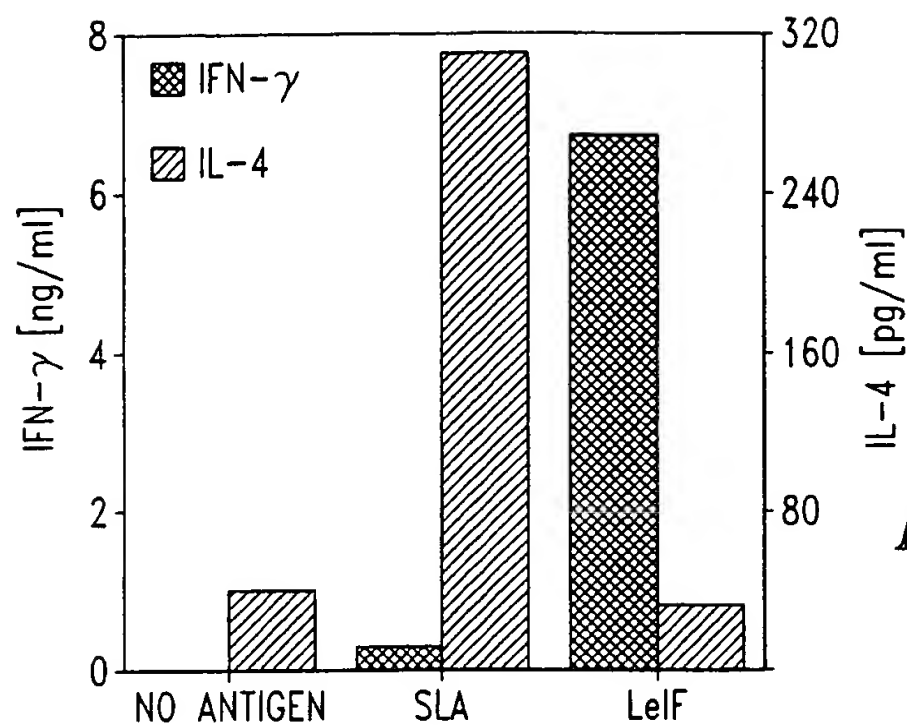
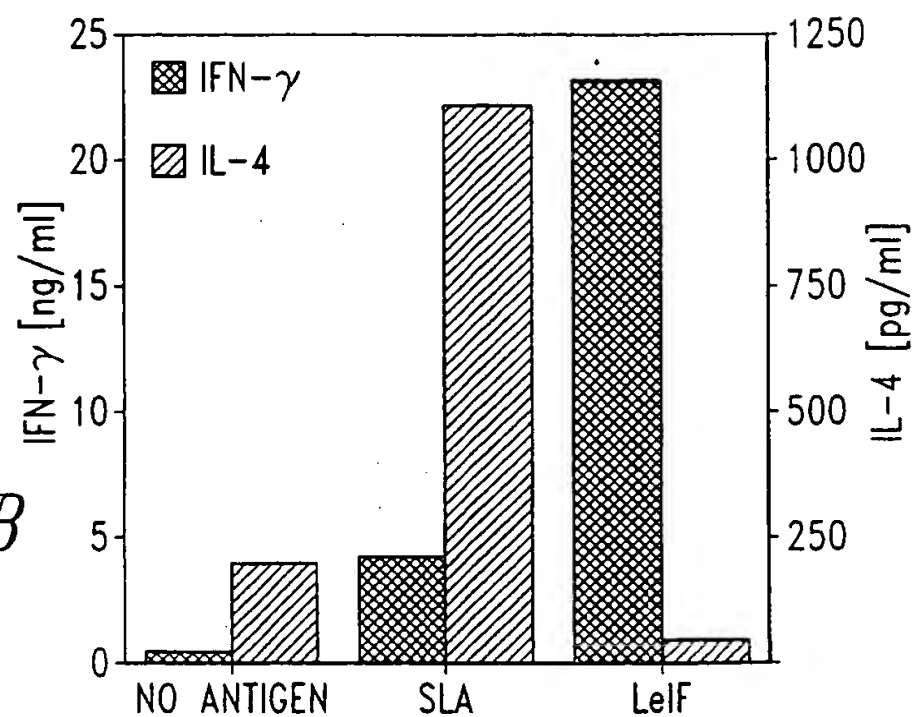
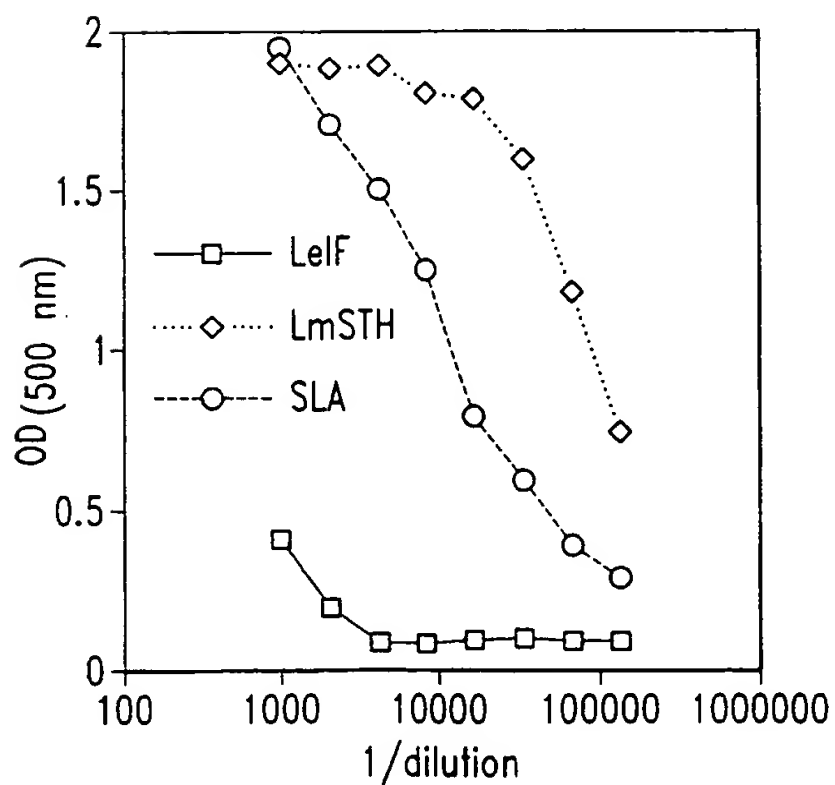
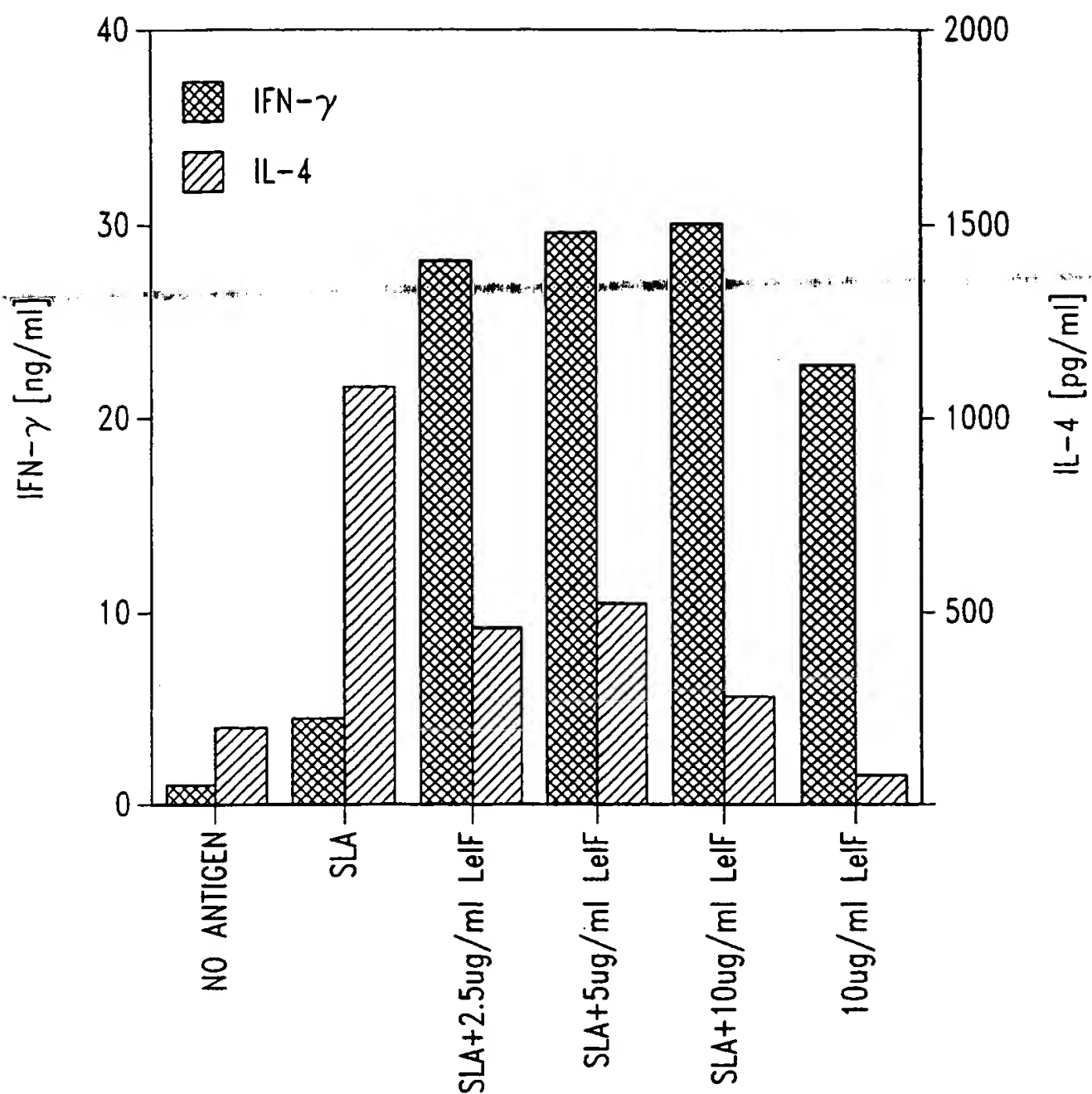


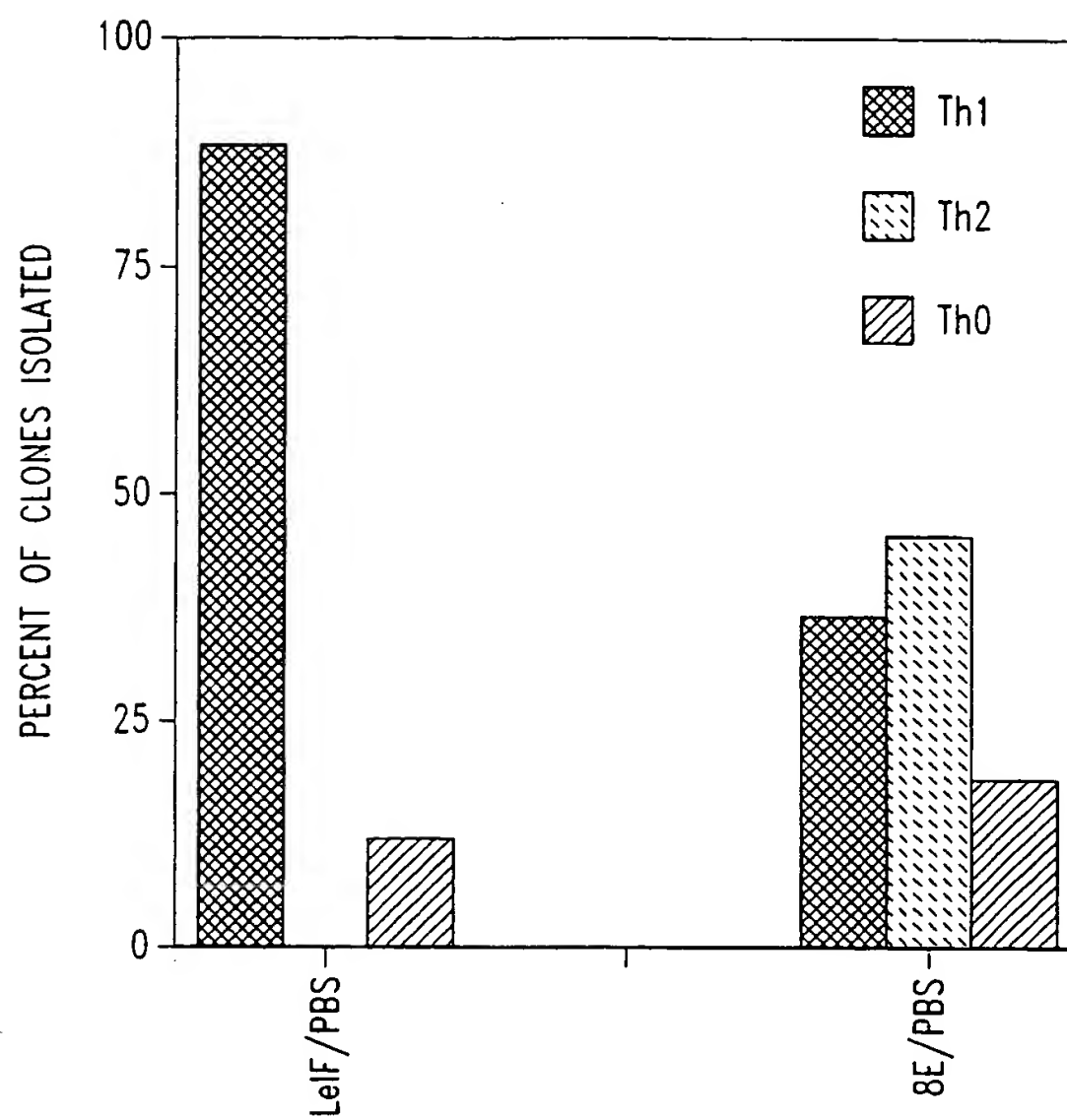
Fig. 23C

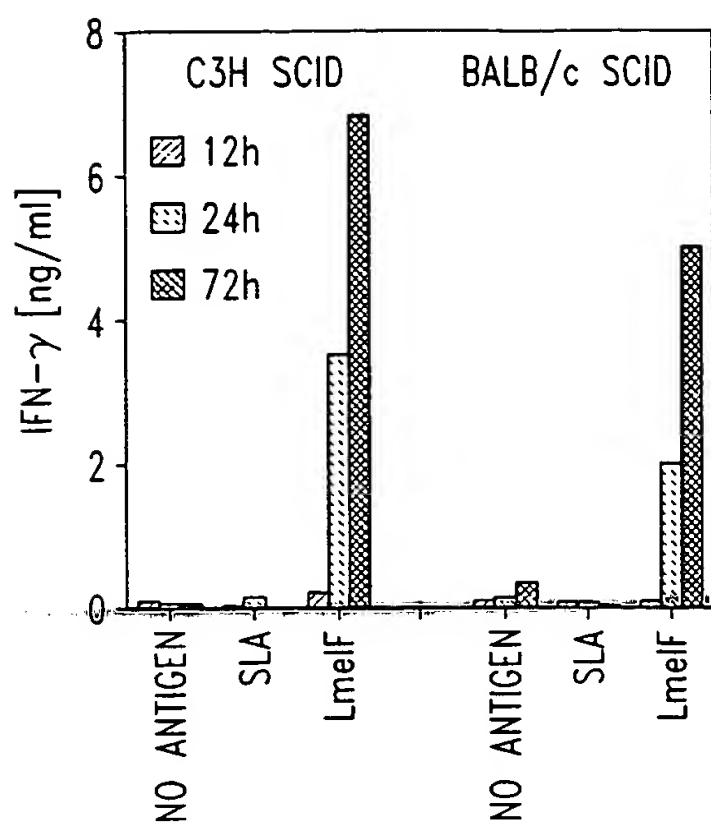
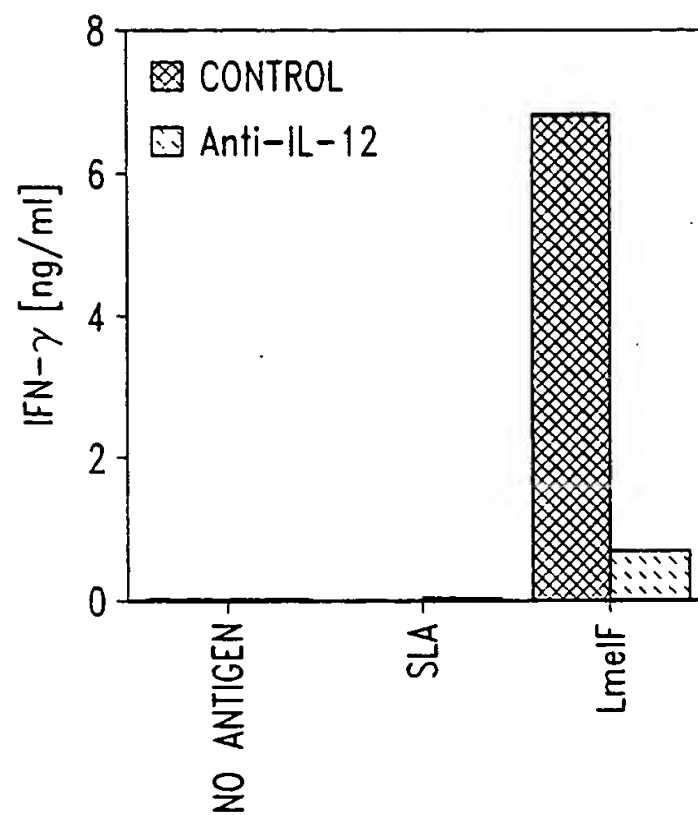
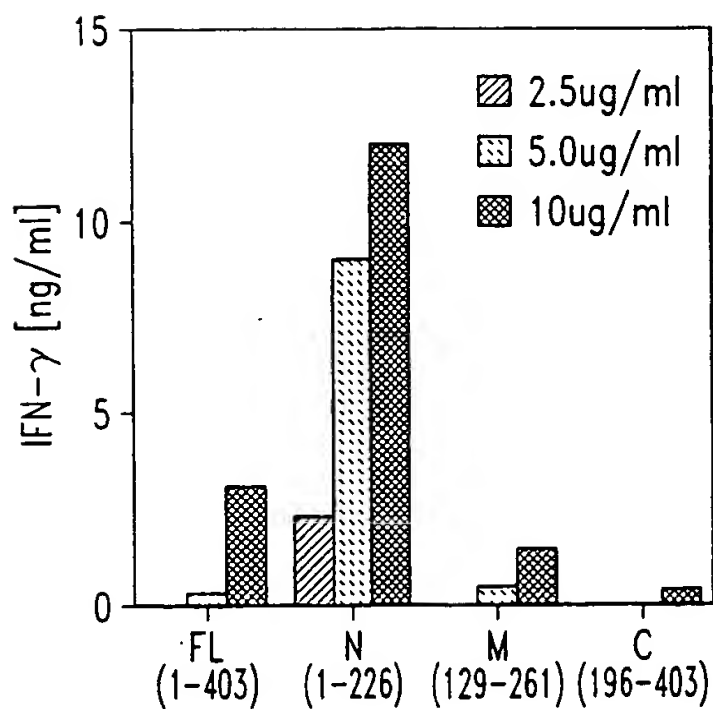


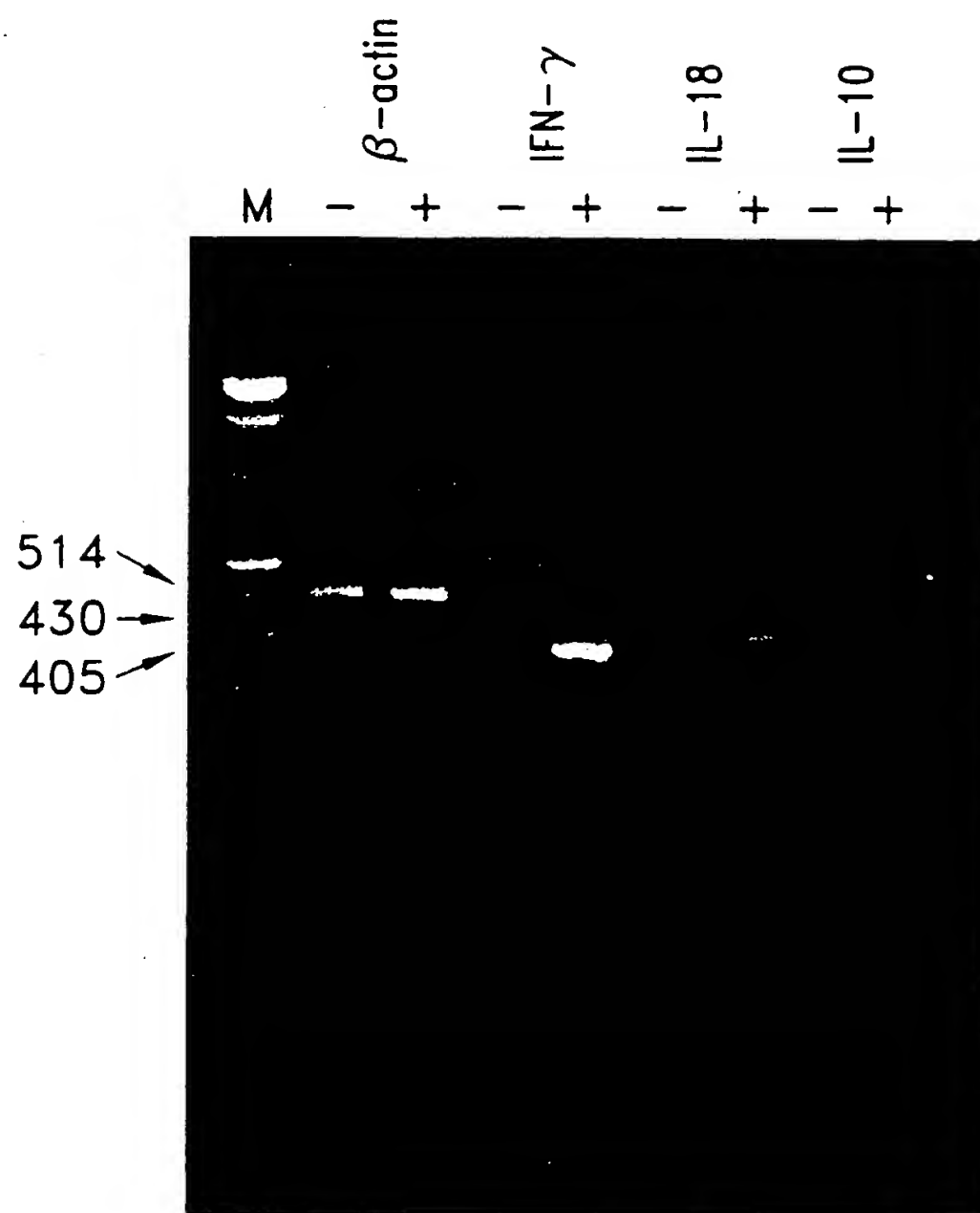
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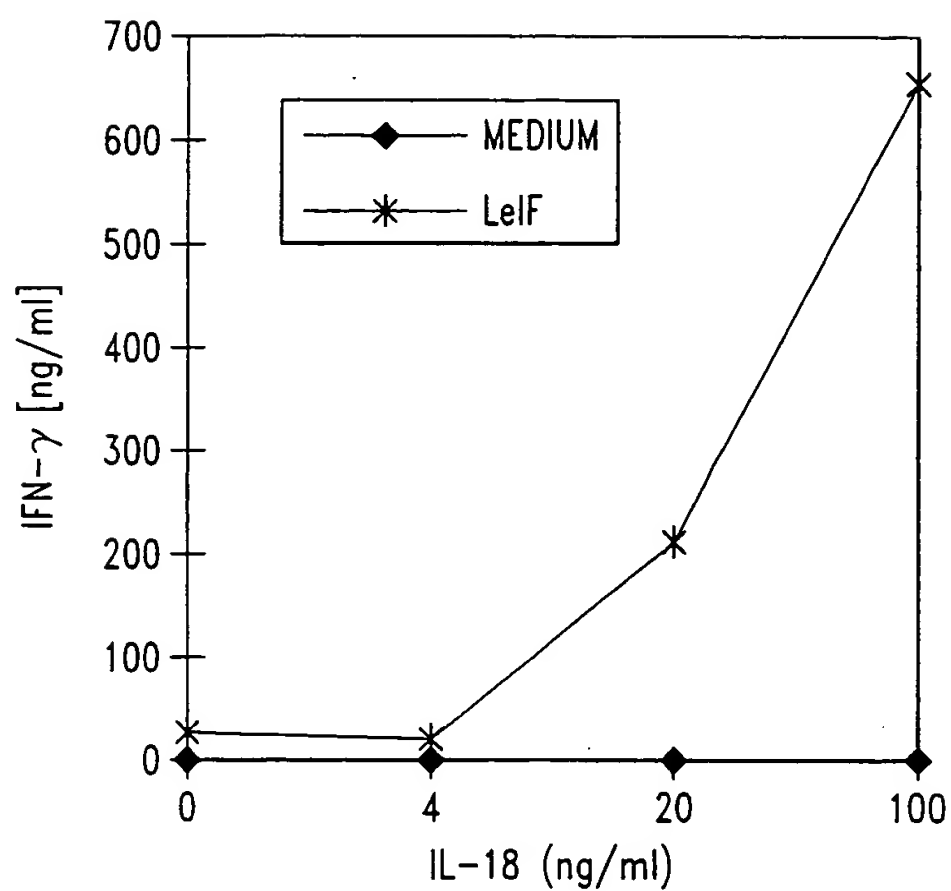
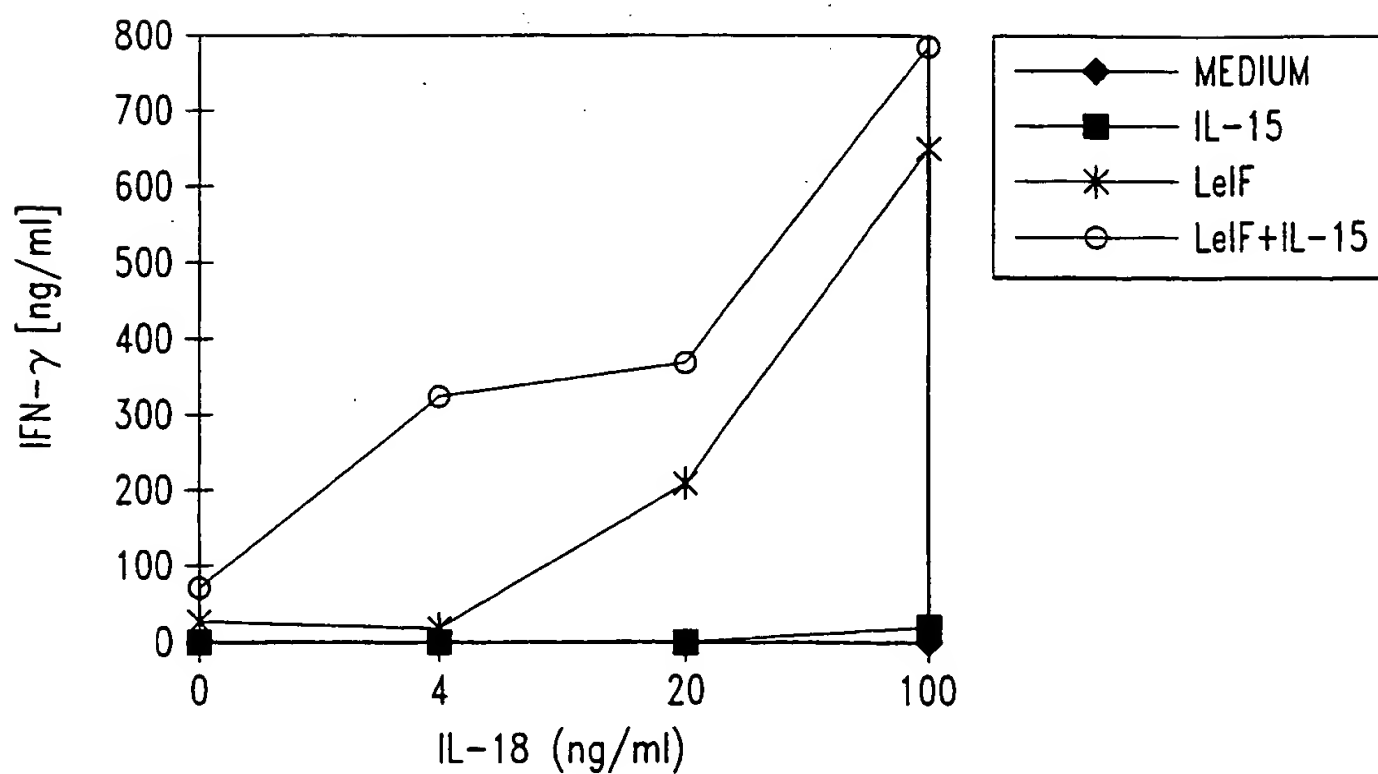
*Fig. 24A**Fig. 24B**Fig. 24C*

*Fig. 25*

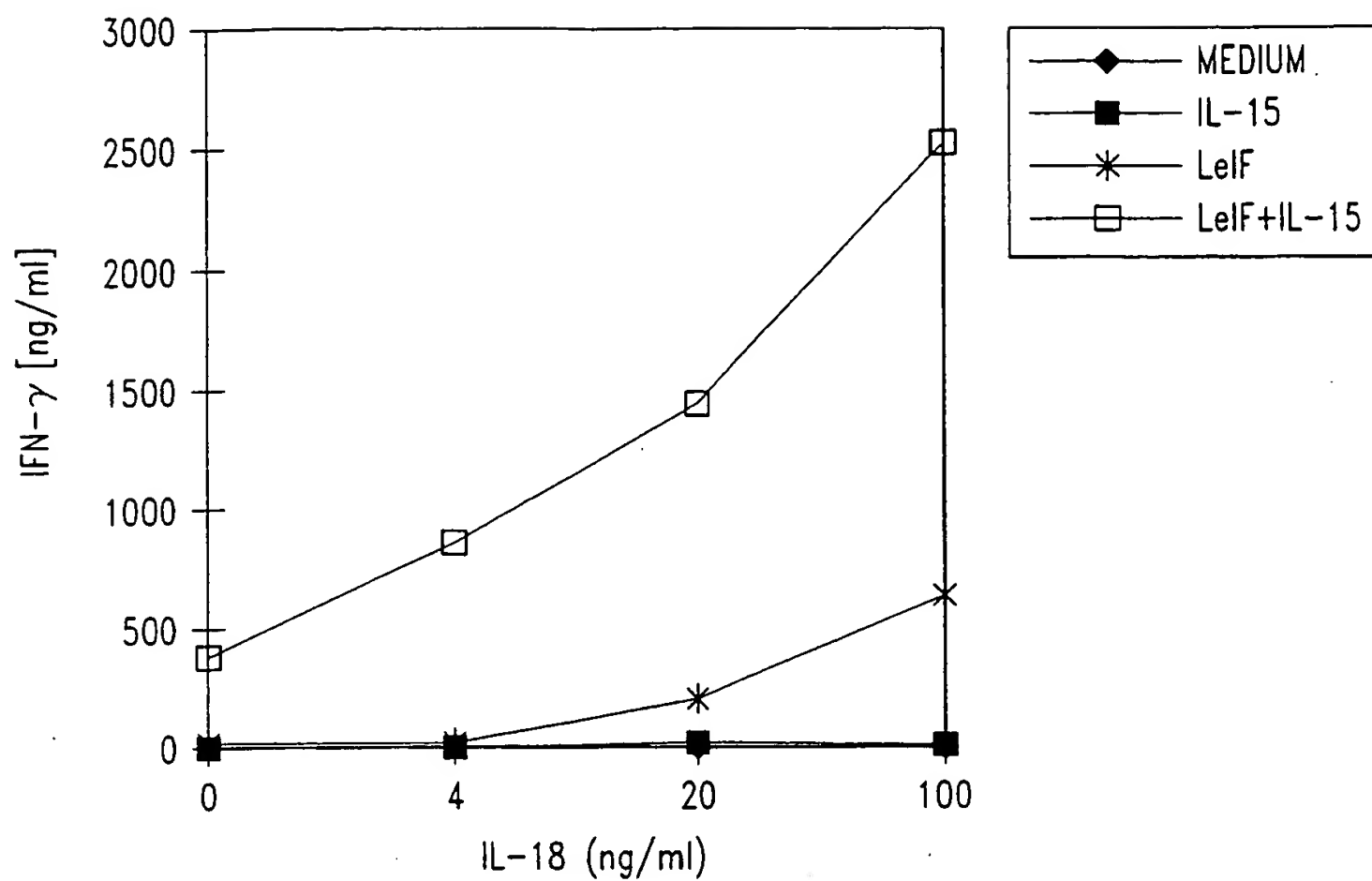
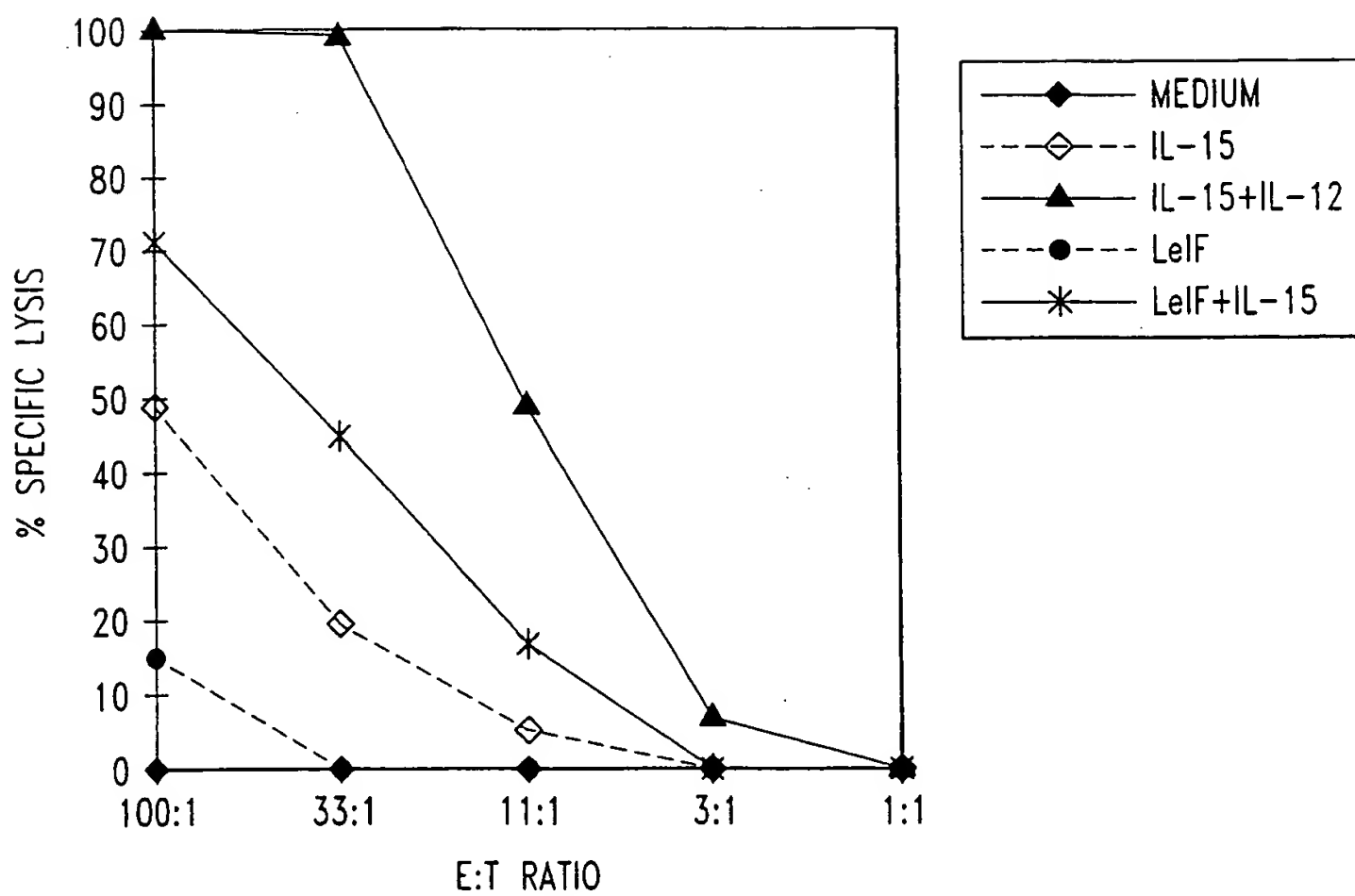
*Fig. 26*

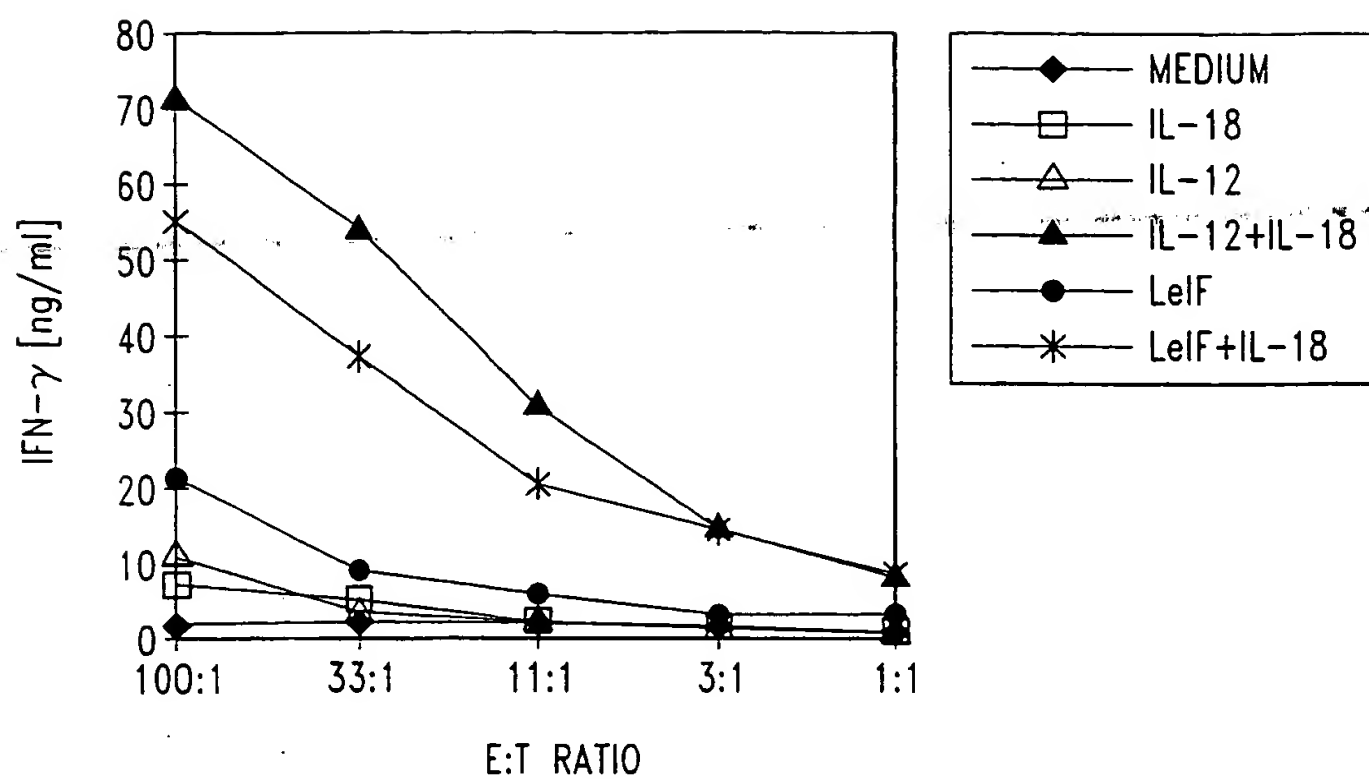
*Fig. 27A**Fig. 27B**Fig. 27C*

*Fig. 28*

*Fig. 29**Fig. 30*

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*Fig. 31**Fig. 32*

*Fig. 33*





## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 39/008, C07K 14/44, C12N 15/30</b> <b>// (A61K 39/008, 45:05)</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 99/29341</b> <b>(43) International Publication Date:</b> 17 June 1999 (17.06.99)
<b>(21) International Application Number:</b> PCT/US98/26438 <b>(22) International Filing Date:</b> 11 December 1998 (11.12.98) <b>(30) Priority Data:</b> 08/989,370 12 December 1997 (12.12.97) US <b>(71) Applicant:</b> CORIXA CORPORATION [US/US]; Suite 200, 1124 Columbia Street, Seattle, WA 98104 (US). <b>(72) Inventor:</b> REED, Steven, G.; 2843 - 122nd Place Northeast, Bellevue, WA 98005 (US). <b>(74) Agents:</b> MAKI, David, J. et al.; Seed and Berry LLP, 6300 Columbia Center, 701 Fifth Avenue, Seattle, WA 98104-7092 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims</i> <i>and to be republished in the event of the receipt of amendments.</i>  <b>(88) Date of publication of the international search report:</b> 28 October 1999 (28.10.99)
<b>(54) Title:</b> METHODS FOR ENHANCEMENT OF PROTECTIVE IMMUNE RESPONSES EMPLOYING <i>LEISHMANIA</i> POLYPEPTIDES  <b>(57) Abstract</b>  Methods for eliciting or enhancing immune responses to antigens, including tumor antigens, and/or DNA vaccines are provided. The methods employ polypeptides or nucleic acid compositions that contain at least a biologically active portion of a <i>Leishmania braziliensis</i> or <i>Leishmania major</i> homologue of the eukaryotic initiation factor 4A, or a variant thereof. Such polypeptides and compositions are useful for enhancing or eliciting a patient's cellular and/or humoral immune response, for instance within methods for treating tumors.		

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/26438

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K39/008 C07K14/44 C12N15/30  
 //(A61K39/008,45:05)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 39524 A (CORIXA CORP) 12 December 1996 (1996-12-12) the whole document	73-77
P,X	<p style="text-align: center;">---</p> <p>SKEIKY, YASIR A. ET AL: "LeIF: a recombinant Leishmania protein that induces and IL-12-mediated Th cytokine profile"</p> <p>J. IMMUNOL. (1998 DECEMBER 1), 161(11), 6171-6179 CODEN: JOIMA3;ISSN: 0022-1767, XP002103156</p> <p>page 6171</p> <p>page 6178 - page 6179, left-hand column</p> <p>figures 1-3,5-7</p> <p style="text-align: center;">-----</p>	73-77



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents :

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- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search

19 May 1999

Date of mailing of the international search report

10. 09. 99

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/ 26438

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos..  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos..  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos..  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

73 - 77

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

## 1. Claims: 73-77

Claim 73 is directed to an LeIF4A polypeptide or a nucleic acid molecule, such as a viral vector, directing the expression of the same for use in the manufacture of a medicament for treating a Th2-mediated disease.

Claim 74 is dependent on claim 73.

Claim 75 is directed to a LeIF4A polypeptide or a nucleic acid molecule, such as a viral vector, directing the expression of the same for use in the manufacture of a medicament for decreasing production of one or more Th2-associated cytokines in a patient.

Claims 76 and 77 are each dependent on claim 75.

## 2. Claim : 78

Claim 78 is directed to an LeIF4A polypeptide or a nucleic acid molecule, such as a viral vector, directing the expression of the same, for use in the manufacture of a medicament for stimulating or enhancing IL-18 (which is a Th1-associated cytokine) production in a patient.

## 3. Claims: 79-82

Claim 79 is directed to a method for enhancing or eliciting an immune response to an antigen in a biological sample, comprising contacting the sample with a LeIF4A polypeptide in combination with one or more Th1-associated cytokines.

Claim 80 is dependent on claim 79.

Claim 81 is directed to a composition comprising one or more Th1-associated cytokines in combination with a LeIF4A polypeptide or nucleic acid molecule, such as a viral vector, directing the expression of the same, for use in the manufacture of a medicament for enhancing or eliciting an immune response to an antigen.

Claim 82 is dependent on claim 81.

## 4. Claims: 1-72

Claims 1-14 are directed to compositions comprising an antigen and a LeIF4A polypeptide (LbeIF4A for cl. 1-4, 9 and

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

11-14 [part]; LmeIF4A for cl. 5-8, 10 and 11-14 [part]) for use in the manufacture of a medicament for enhancing or eliciting an immune response to an antigen.

Claims 15-28 are directed to compositions comprising a DNA vaccine and a LeIF4A polypeptide (LbeIF4A for cl. 15-18, 23 and 25-28 [part]; LmeIF4A for claims 19-22, 24 and 25-28 [part]) for use in the manufacture of a medicament for enhancing or eliciting an immune response to a DNA vaccine.

Claims 29-30 are directed to LeIF4A polypeptides (LbeIF4A for cl. 29; LmeIF4A for cl. 30) for use in the manufacture of a medicament for enhancing or eliciting an immune response to an antigen.

Claim 31-36 are directed to methods of enhancing or eliciting an immune response in a biological sample comprising contacting the sample with an antigen and a LbeIF4A polypeptide that stimulates a Th1 immune response, wherein the sample comprises cells selected from the group consisting of peripheral blood mononuclear cells, monocytes, B cells, dendritic cells and combinations thereof.

Claims 37-42 and 51 are directed to compositions comprising a tumor antigen and a LeIF4A polypeptide (LbeIF4A for cl. 37-41 and 51; LmeIF4A for cl. 42) for use in the manufacture of a medicament for enhancing or eliciting an immune response to a tumor.

Claims 43-48 are directed to compositions comprising a DNA vaccine encoding a tumor antigen and a LeIF4A polypeptide (LbeIF4A for claims 43-47; LmeIF4A for claim 48) for use in the manufacture of a medicament for enhancing or eliciting an immune response to a tumor.

Claims 49-50 and 52 are directed to LeIF4A polypeptides (LbeIF4A for cl. 49 and 52 [part]; LmeIF4A for cl. 50 and 52 [part]) for use in the manufacture of a medicament for treating a tumor.

Claims 53-64 (and dependent claims 65-72) are directed to compositions comprising a nucleic acid molecule directing the expression of a LbeIF4A or LmeIF4A polypeptide for use in the manufacture of a medicament for enhancing or eliciting an immune response to an antigen (cl. 53-58) or a tumor (cl. 59-64). In claims 53, 55, 57, 59, 61 and 63 the nucleic acid molecule is a viral vector. Compositions of claims 53-56 comprise additionally an antigen (cl. 53-54, 59 and 60) or a DNA vaccine (cl. 55-56, 61 and 62).

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/26438

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9639524 A	12-12-1996	US 5876735 A	02-03-1999
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